CHARACTERIZING THE BIOLOGY AND EPIDEMIOLOGY OF FUNGICIDE-RESISTANT *VENTURIA EFFUSA* FOR IMPROVED PECAN SCAB MANAGEMENT

by

JEFFREY RUSSELL STANDISH

(Under the Direction of Timothy Brenneman and Katherine Stevenson)

ABSTRACT

Pecan scab, caused by *Venturia effusa*, is the most economically damaging disease affecting pecan (*Carya illinoinensis*) in the southeastern United States. Managing scab requires fungicides and is often the greatest operating cost for commercial growers. The work detailed here aimed to characterize the biology of fungicide-resistant *V. effusa* with the goal of improving fungicide use patterns throughout Georgia pecan orchards. An intron downstream of position 143 in the *V. effusa* cytochrome *b* gene was identified in all sequenced isolates from these studies indicating that the risk of quinone outside inhibitor resistance is relatively low. A novel glycine to serine amino acid substitution was identified in a subset of 14 isolates and a detached leaf assay was developed to screen sensitivity of isolates to azoxystrobin. Isolates with G137S were less sensitive than wild-type isolates. The relationship between fungicide sensitivity determined using in vitro bioassays and fungicide efficacy in the field was explored but a consistent quantitative relationship was not observed. Sensitivity and efficacy results obtained from fentin hydroxide reveal that relative insensitivity values between 0.6 and 40.9% could

occur without causing a likely control failure, although efficacy was reduced compared with other treatments. Interestingly, when tebuconazole insensitivity values were between 34.6 and 69.3%, a control failure was likely on trees treated with tebuconazole. Spatial and temporal dynamics of fungicide sensitivity were examined in a commercial orchard over 3 years. Sensitivity values differed in each year and were spatially dependent for propiconazole and thiophanate-methyl in 2017, but in no other instance. A separate study revealed that in most cases, a sample size of three groups of leaflets to be sufficient for sensitivity testing, although a fourth group could improve precision. Fitness attributes were compared, and phenotypic stability was assessed between isolates sensitive and insensitive to fentin hydroxide and tebuconazole. Conidial germination was negatively correlated with fentin hydroxide insensitivity, and tebuconazole insensitivity was negatively correlated with hypersensitivity to 1.0 mM H_2O_2. Tebuconazole sensitivity did not differ over consecutive generations but fentin hydroxide sensitivity did, indicating that insensitivity is likely a temporary adaptation of the pathogen to the fungicide.

INDEX WORDS: *Ventura effusa*, pecan, pecan scab, cytochrome *b* gene, amino acid substitution, G137S, fungicide resistance monitoring, sampling, spatial autocorrelation, fitness cost, resistance stability

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DEDICATION

To my wife, Marianne.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Pecan background, history, and production

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is a deciduous tree within the family Juglandaceae and the most economically important of the 20 known species of hickory (Rüter et al. 1999). Growth habits of pecan are characterized as being tall and straight with a uniform, symmetrical, and broadly oval crown. Pecan will typically grow 21 to 30 m in height with a spread of 12 to 22 m (Dirr 1998). Trees develop a long taproot and prefer deep, moist, and well-drained soils not prone to prolonged periods of flooding (Dirr 1998). Leaves are alternate, pinnately compound, 30 to 50 cm long and composed of 11 to 17 oblong-lanceolate leaflets, each measuring between 10 and 43 cm long by 2 to 7 cm wide (Dirr 1998). Flowers are unisexual, with both sexes borne in clusters at different locations on the same tree. Pistillate flowers are borne terminally on the shoot while staminate flowers develop as slender catkins of 7 to 12 cm in length at the base of emerging shoots having aborted shortly after bud break (Sparks 1992). The staminate flowers produce a large quantity of pollen, which is transported by wind to the stigmatic surface of the pistillate flowers. Pecan cultivars typically exhibit dichogamy, where pollen shedding intervals do not closely coincide with stigma receptivity (Sparks 1992). This characteristic helps to prevent self-pollination, which may lead to nut abortion or poor kernel quality (Wells 2007). Cultivars differ in their annual occurrence of pollen shed and stigma receptivity, so planting at least two cultivars with

complementary flowering characteristics in the same orchard ensures pollination (Wells 2007). The fruit form in clusters of 3 to 12 and consist of three parts: the kernel, shell, and shuck. The kernel is the edible portion of the fruit and is found within the shell, which is housed within the shuck (Sparks 1992). The kernel consists of two cotyledons referred to as halves, which are separated by the central partition wall within the shell. The shuck is made up of four quarters or sections that split at maturity to expose the nut (Sparks 1992).

As a native North American tree species, pecan could originally be found from Iowa to Indiana to Alabama, Texas, and Mexico along the floodplains of the Mississippi, Missouri, and Ohio rivers; the Red rivers and their tributaries; and along many of the larger rivers of eastern Texas and northeastern Mexico (Dirr 1998; Wood et al. 1990). Pecans were highly desirable to the native inhabitants of pre-colonial North America as they were regarded as having the best taste of the native hickory species and were the easiest to shell. The nuts were readily available due in part to their relative proximity to major waterways (Wood et al. 1990). Cultivation of pecan began in the mid to late 1800's as vegetative propagation techniques were perfected to create improved cultivars. Many of the early cultivars produced during this time were failures; however, the early breeders also created some of the most successful cultivars grown today such as Desirable, Schley, Stuart, and Western Schley (Sparks 1992). At the turn of the 20th century, many existing seedling orchards were top-worked with the hopes of establishing improved cultivars that would produce large and easy-to-shell nuts (Wood et al. 1990). During this time period, large numbers of orchards were planted in southwestern Georgia and east-central Texas. This began a shift in the major production region away from

eastern Texas, Louisiana, and southern Mississippi to southwestern Georgia (Wood et al. 1990).

The United States and Mexico rank number one and two, respectively, in the world for pecan production, while Australia, Brazil, Israel, Peru, and South Africa all produce smaller pecan crops (Perez and Pollack 2003). In 1909, U.S. pecan growers produced approximately 4,500 metric tons of pecan and by 1939, U.S. production had grown to greater than 33,000 metric tons (NASS 1940). Total pecan production in the U.S. exceeded 133,000 metric tons in 2017 with a value of approximately $684.3 million (NASS 2018). Pecans are produced in 14 states with Georgia accounting for about one-third of total U.S. annual production on average. Georgia pecan growers produced approximately 42,000, 49,000, and 48,000 metric tons during the 2015, 2016, and 2017 growing seasons, respectively. Over the same three years, New Mexico ranked second among U.S. pecan producing states with 33,000, 32,000, and 41,000 metric tons produced, respectively; Texas, Arizona, and Oklahoma rounded out the top five (NASS 2018).

Pecan scab and *Venturia effusa*

Pecan scab, caused by the fungus *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), is the most destructive disease affecting pecan (Demaree 1924). Scab was first reported in 1882 by F. S. Earle, who collected the type specimen from affected leaves of Mockernut hickory [*Carya tomentosa* (Lam.) Nutt.] in Illinois (Demaree 1928). The collection was sent to Berlin, Germany where the isolates were first described as *F. effusum* by G. Winter in 1885 (Demaree 1928). Demaree (1924) identified that high humidity as well as high temperatures were essential for pecan

scab development. Initially, scab was typically observed affecting orchards within a 100-mile distance inland from the Gulf of Mexico; after which, the incidence and severity of the disease appeared to decrease (Demaree 1924). However, due to an increase in the number of plantings and frequent summer rains, the range of scab distribution began to extend inward and away from the Gulf of Mexico (Demaree 1924). Today, scab occurs in east Texas but does not occur throughout the Southwest into California. The annual severity of scab is determined by the relative abundance of *V. effusa* inoculum and frequency of spring and summer rainfall; pecan scab is typically most damaging in the humid southeastern U.S. (Latham 1982).

Symptoms. Pecan scab occurs as lesions on leaves, fruit shucks, and twigs. Young and actively growing tissues are most susceptible to infection but become resistant as they mature (Demaree 1924; Littrell and Bertrand 1981). Infected leaves exhibit small olive-brown to black spots, approximately 1 to 5 mm in diameter that may expand and coalesce to form large, irregularly-shaped lesions (Fig. 1.1A). When the fungus sporulates, lesions have a velvety or rough appearance and most commonly appear on the underside of the leaf surface (Demaree 1924; Bertrand 2002). Scab occurs on current-season twigs as small, olive-brown to black colored spots of approximately 0.5 to 3 mm in diameter (Demaree 1924). Shuck infections between fruit growth and shell hardening cause the greatest damage. Small, circular lesions develop and slowly enlarge, up to 12 mm in diameter, although most spots reach 3 mm and stop expanding (Fig. 1.1B). Lesions are typically olive-brown to gray when first visible and become olive-brown with a gray border, generally exhibiting a jagged, irregular, and indefinite outline (Demaree 1924; Bertrand 2002). Severe fruit infection may cause complete termination of fruit

development; however, infection after the shell has hardened is thought to be more cosmetic than damaging (Demaree 1924; Gottwald and Bertrand 1983; Hunter 1983).

Epidemiology. Pecan scab follows a polycyclic disease cycle in which inoculum of *V. effusa* may develop over several generations in the same growing season. The fungus overwinters as black stromata within lesions on leaves, shoots, and shucks (Demaree 1924). Dark brown conidiophores are produced directly from stromata and give rise to long chains of two to nine light brown single-celled conidia (Fig. 1.2) (Demaree 1928). Conidia produced from an overwintered stroma are thought to act as the primary inoculum for pecan scab epidemics as conidia are present while young leaves are unfolding and most susceptible to infection (Demaree 1924). A rapid decrease in relative humidity, combined with an increase in temperature and infrared radiation are factors that lead to an increase in the concentration of air-borne conidia (Gottwald 1982; Gottwald and Bertrand 1982). Dispersal of conidia occurs during daylight hours by wind and splash (Gottwald 1982; Gottwald and Bertrand 1982; Latham 1982). Continuous foliar moisture for 12 to 48 h coupled with temperatures between 15 and 25°C provide the optimal conditions that allow for conidial germination and infection to occur (Converse 1960; Demaree and Cole 1929; Gottwald 1985; Turechek and Stevenson 1998). Symptoms will develop within 5 to 21 days upon infection, provided conducive environmental conditions persist; conidia formed from these new infection sites act as secondary inoculum and are produced in abundance for the majority of the growing season (Demaree 1924; Latham 1982; Littrell and Bertrand 1981). The sexual stage of *V. effusa* has never been observed in nature; however, a sexual structure (a pseudothecium producing ascospores) has been observed in vitro (Charlton et al. 2016). Addtionally,

great genetic diversity has been observed in populations of *V. effusa* and the pathogen exists in mating-type equilibrium indicating that such a cycle may be occurring at some time in the field (Bock et al. 2017; Young et al. 2018).

Management. The most effective method for managing scab is to plant resistant cultivars (Conner and Wells 2007). However, the scab pathogen exhibits great genetic and pathogenic diversity allowing for its adaptation to cultivars with scab resistance; many older varieties were thought to be immune to scab but are now considered highly susceptible after consistent exposure to the diverse scab population over time (Bock et al. 2014, 2017; Conner and Stevenson 2004; Demaree and Cole 1929; Sparks 1992). In the absence of varietal resistance, the most effective scab control measures are to make multiple preventive fungicide applications (Brock and Bertrand 2007a). Fungicide programs for disease control in pecan are centered on the need to control pecan scab because a complete crop failure may occur (Littrell and Bertrand 1981). Crop losses in the state of Georgia as caused by scab in 2010, 2011, 2012, 2013, 2014, and 2015 were estimated to be 2.0, 1.0, 1.5, 15.0, 10.0, and 12.0%, respectively. During the same years, the estimated total combined cost of damage and control measures were $25.4, 23.7, 27.3, 78.7, 25.7, and 65.0 million, respectively (Brock 2012; Brock and Brenneman 2013; 2015a; 2015b; 2016; 2017).

Chemical disease control

The general guideline for pecan scab prevention in Georgia calls for 7 to 10 fungicide applications per season, made on 10- to 14-day intervals from bud break until pollination; and on 14- to 21-day intervals from pollination to shell hardening (Brock and Bertrand 2007b). Early season applications are typically made to delay the initial onset

of scab symptoms on susceptible leaf and shoot tissue but are continued on the above-mentioned intervals to help halt disease progress. Similarly, late-season applications are made to delay the onset of nut scab symptoms; infections occurring early in the season have been shown to have a greater impact on yield compared to those occurring closer to harvest (Gottwald and Bertrand 1983; Stevenson and Bertrand 2001). During rainy periods, the interval between sprays may be adjusted to every 7 to 10 days, which has the potential to substantially increase the total number of applications per season (Latham 1995). The scheduling of these preventive applications depends on the environmental conditions during a given growing season and can be modified to fit the needs of a given orchard (Brock and Bertrand 2007b). In addition to the calendar-based spray schedule described above, fungicide applications may also be scheduled using predictive models that utilize weather data (Brenneman et al. 1998; Brock and Bertrand 2007b; Payne and Smith 2012). The weather-based spray advisory system provides guidance for specific timing of fungicide applications, which in a dry year may lead to fewer applications when compared with a calendar-based system (Brenneman et al. 1998). Fungicides from different chemical groups are approved for use on pecan in the U.S. and include the guanidines, organotin compounds, methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and phosphonates, among others (Fungicide Resistance Action Committee [FRAC] Code U12, 30, 1, 3, 11, and 33, respectively) (Bock et al. 2012; Brock et al. 2007).

Dodine is the only chemical within the guanidine group and was first introduced in 1956 as a protectant fungicide (Brock and Brenneman 2015c; Sisler and Ragsdale 1981). First applied for scab management in 1963, dodine has multi-site activity with an

unknown mode of action that is thought to involve the disruption of cell membranes (Sisler and Ragsdale 1981). Dodine is recommended as a tank-mix partner with other fungicides for pre-pollination scab management. The post-pollination recommendation follows the same guideline with dodine used as a mixing partner, with the exception that stand-alone applications may also be made (Brock and Brenneman 2015c).

Fungicides in the organotin group are applied in a protectant manner to control a number of plant diseases (Sisler and Ragsdale 1981). The triphenyltins have a non-specific, multi-site mode of action that has not been completely characterized. The currently accepted mode of action involves the inhibition of oxidative phosphorylation by catalyzing an anion-hydroxide exchange across the inner mitochondrial membrane, binding there, and inhibiting ATP synthase and ATPase (Ayoko et al. 2003; FRAC 2016a; Sisler and Ragsdale 1981; von Ballmoos et al. 2004). Fentin hydroxide, also known as triphenyltin hydroxide (TPTH), is an organotin fungicide that was first labelled for use on pecan in 1967 (Brock and Brenneman 2015c; Littrell and Bertrand 1981). Current recommendations for use of fentin hydroxide for management of both pre- and post-pollination scab mirror those of dodine as previously mentioned (Brock and Brenneman 2015c).

The MBC fungicides are a group of systemic compounds that interfere with nuclear division by binding to the β-tubulin subunit and inhibiting microtubule production. By suppressing the function of microtubules, the MBCs can effectively disrupt mitosis during spore germination and hyphal growth (Davidse 1973; Davidse 1986; Davidse and Flach 1978; Ishii 1992; Hollomon et al. 1998; Sisler and Ragsdale 1981). Thiophanate-methyl is labelled for use on pecan as a tank-mix partner of either

TPTH or dodine but is not recommended for use as a stand-alone product (Brock and Brenneman 2015c; Stevenson 1998).

The DMI group of fungicides target the sterol 14α-demethylase CYP51, an important enzyme in the ergosterol biosynthetic pathway. These fungicides render the CYP51 catalytically inactive, which prevents the demethylation of lanosterol and eburicol thereby inhibiting the production of ergosterol, which is necessary to maintain fungal membrane fluidity and permeability (Köller 1992; Price et al. 2015). This process leads to the disruption of membrane structure and prevents active membrane transport due to a combination of two factors: the depletion of ergosterol in the cell and the accumulation of 14α-demethylated sterols; resulting in fungistasis (Price et al. 2015). Stand-alone DMI fungicides labelled for use on pecan in the U.S. are propiconazole, fenbuconazole, tebuconazole, tetraconazole, and metconazole. The DMIs are labelled for pre-pollination pecan scab management applications as stand-alone formulated mixtures or tank-mix partners with a QoI, dodine, or TPTH. Post-pollination applications are made as tank mix partners with the three previously mentioned chemicals (Brock and Brenneman 2015c).

The QoI fungicides inhibit mitochondrial respiration by binding to the quinol oxidation site of the cytochrome bc_1 enzyme complex, blocking electron transfer between the cytochrome *b* (cyt *b*) and cytochrome c_1; a process that halts the production of ATP resulting in an energy deficiency in fungal cells (Bartlett et al. 2002; Fernández-Ortuño et al. 2008; Gisi et al. 2002). These fungicides are major tools in scab management both as stand-alone products and as premix combinations, most often with a DMI. There are several stand-alone QoI fungicides commercially available for use on pecan in the U.S.,

including azoxystrobin, kresoxim-methyl, and pyraclostrobin. Additionally, pre-mix combinations of a QoI and a DMI fungicide are also currently available for scab control in commercial pecan production. These include formulated mixtures of azoxystrobin and propiconazole, azoxystrobin and difenoconazole, azoxystrobin and tebuconazole, and trifloxystrobin and tebuconazole (Brock and Brenneman 2015c). For pre-pollination scab management, the QoIs may be applied as stand-alone products or as tank mix partners with DMIs; for post-pollination scab management, tank mixtures of a QoI and a DMI are recommended (Brock and Brenneman 2015c).

Fungicides within the phosphonates group are particularly efficacious against oomycete pathogens as well as certain fungal pathogens, including *V. effusa* (Bock et al. 2012, 2013a; Fenn and Coffey 1984; Jackson et al. 2000; Miller et al. 2006; Ouimette and Coffey 1989; Percival et al. 2009). The mode of action for this group is poorly understood but they seem to protect the plant in two distinct ways (FRAC 2016a). First, the fungicide may act directly on the pathogen to inhibit growth before infection can occur; and second, the fungicide may act indirectly by stimulating host defense enzymes prior to infection (Bock et al. 2013a; Fenn and Coffey 1984; Guest and Grant 1991; Jackson et al. 2000; Saindrenan et al. 1988). There are several commercial products within this group that are labelled for use in controlling scab; all of which contain phosphorous acid as the active ingredient (Brock and Brenneman 2015c).

Fungicide resistance

Fungicide resistance is the acquired and heritable reduction in sensitivity of a fungus to a fungicide that occurs because of the selection of insensitive members within a population (FRAC 2016b). Practical resistance is the phenomenon occurring when a

population has shifted to one that is predominantly resistant, leading to disease control failures after application of the recommended dose of the fungicide. Practical resistance may develop in either a quantitative or qualitative manner (Brent and Hollomon 2007; FRAC 2016b). Quantitative or multi-step resistance is a process where the accumulation of multiple mutations at the target site results in a gradual shift from sensitivity to insensitivity in the pathogen population over several years (Brent and Hollomon 2007; FRAC 2016b). Conversely, qualitative or single-step resistance occurs as the result of a single mutation in the gene that encodes the target site that results in development of two sub-populations. These two populations have very different sensitivities to a given fungicide and the sudden loss of product efficacy will follow (Brent and Hollomon 2007; FRAC 2016b). The likelihood of resistance occurring depends on biological (i.e., pathogen), chemical (i.e., fungicide), and agronomic (i.e., environment) factors (FRAC 2013; Kuck 2005). For the pathogen, there are many properties that influence the evolution and spread of resistance, but not all pathogens have the same risk. The method of propagule dispersal, abundance of inoculum in time and space, and pathogen life cycle (monocyclic vs. polycyclic), are all factors influencing how prone a pathogen is to resistance developing (Hollomon 2015a). Additionally, several genetic factors also play a role, including the relative abundance of individuals with differing sensitivities, competitive ability of different genotypes, and the influence of sexual or asexual reproduction on inheritance of resistance (Hollomon 2015a). For the fungicide, several factors influence the likelihood of resistance occurring; similar to the pathogen, not all fungicides have the same risk. Mode of action is the major factor; a chemical with a single target site has a higher risk for resistance to develop in a population when

compared to one that affects multiple sites (FRAC 2013; Hollomon 2015a). Dose and spray coverage, formulation, and the exclusive or repeated use of at-risk fungicides can also contribute to a fungicide's risk for resistance (Hollomon 2015a). The agronomic risk associated with fungicide resistance summarizes the environmental and cultural factors that influence disease severity (Kuck 2005). Weather conditions, fertilization, irrigation, tillage practices, crop rotation, the use of resistant cultivars, and other sanitary measures may have an impact on primary inoculum levels and pathogen development (Kuck 2005). Thus, if fungicide resistance were to occur, the probability would be greater for a field or area with a higher agronomic risk (Kuck 2005).

As previously mentioned, there are many fungicides labelled for use on pecan to manage scab. The inherent risk of resistance developing in *V. effusa* to the active ingredients within each of these fungicide groups ranges from "low to medium" to "high" and is magnified by the inability to provide uniform coverage in larger pecan trees (Bertrand and Brenneman 2001; Bock et al. 2013b, 2015; FRAC 2016a). Similarly, as *V. effusa* survives as stromata on its host from season to season, coupled with several generations of inoculum produced during a single season and the potential for sexual recombination; this pathogen undoubtedly has a high risk for resistance to develop (Demaree 1924; FRAC 2013; Young et al. 2018). Practical fungicide resistance in *V. effusa* was first observed in the mid-1970s in association with control failures of the MBC fungicide benomyl, in Alabama and Georgia (Littrell 1976). Since that time, significant reductions in sensitivity have been reported in *V. effusa* isolates to the organotin fentin hydroxide in vitro and to the DMIs, both in vitro and as observed control failures in some locations (Reynolds et al. 1997; Seyran et al. 2010a; Stevenson et al.

2004, 2015). As fungicides form the backbone of pecan scab management, monitoring fungicide sensitivity and managing the development of resistance are very important to identify issues of resistance as they develop and preserve efficacy.

Fungicides within the MBC group have a "high-risk" for resistance development due to their site-specific mode of action (FRAC 2016a). Practical resistance to the MBCs has been reported in approximately 80 different plant pathogens (FRAC 2018). Resistance is conferred as the result of nucleotide point mutations within the β-tubulin gene that result in amino acid substitutions in the target protein. Substitutions at three positions have been detected in MBC-resistant fungal plant pathogens. A change from glutamic acid to alanine, glycine, or lysine at position 198 (E198A/G/K); phenylalanine to tyrosine at position 200 (F200Y); or leucine to phenylalanine at position 240 (L240F) has been found to confer varying levels of resistance. When a fungus carries one of these substitutions, the affinity for the fungicide to bind to the target site is reduced, potentially resulting in decreased disease control (Albertini et al. 1999; Koenraadt et al. 1992; Lehner et al. 2015; Yarden and Katan 1993). Due to their common mode of action, cross-resistance within this class of chemicals is frequently observed; when a fungus is resistant to one MBC active ingredient, the fungus will automatically be resistant to other MBC active ingredients (Brent and Hollomon 2007; Cunha and Rizzo 2003; FRAC 2016a). Negative cross-resistance is also known to occur in MBC-resistant plant pathogenic fungi where the change to resistance automatically confers a change to sensitivity to the N-phenyl carbamates (FRAC Code 10) (Brent and Hollomon 2007; FRAC 2016a).

In 1975, isolates of *V. effusa* collected from orchards located in three counties in southwest Georgia and one county in Alabama were found to be resistant to the MBC active ingredient benomyl after just 3 years of effective control. Resistant isolates were identified in approximately 25% of orchard samples tested; in most cases, scab management practices in that subset of orchards was based on the heavy use of benomyl (Littrell 1976). While applications of thiophanate-methyl have declined sharply, reduced sensitivity to thiophanate-methyl was still detectable in 2008, when 18% of samples collected from commercial orchards showed such reductions when compared with baseline isolates (Seyran et al. 2010a). Further statewide sensitivity testing in 2014 and 2015 revealed the sensitivity of 97 and 100% of samples to be reduced on the discriminatory concentration of 1.0 µg/ml (K. L. Stevenson, unpublished).

Seyran et al. (2010a) observed reduced sensitivity on a discriminatory concentration of 30.0 µg/ml TPTH in 60% of samples tested in 2008, the cause of which is presumably the widespread use of this fungicide across pecan orchards in Georgia (Seyran et al. 2010a). Likewise, reduced sensitivity was observed in 86 and 72% of samples collected in 2014 and 2015 (K. L. Stevenson, unpublished). The risk for resistance to develop to the triphenyltins is low to medium (FRAC 2016a). However, resistance to TPTH was identified in isolates of *Cercospora beticola* Sacc., causing leaf spot of sugar beet in Greece (Giannopolitis 1978) and isolates from the U.S. were later found to be resistant to TPTH (Bugbee 1995; Campbell et al. 1998; Giannopolitis 1978; FRAC 2016a).

Similar to the mode of action of dodine, the mechanism of resistance to this fungicide is not well understood either (FRAC 2016a). However, the risk of resistance to

dodine is low to medium, but practical resistance has been reported in the closely related apple scab pathogen, *Venturia inaequalis* (Cooke) G. Winter (Carisse and Jobin 2010; Jones and Walker 1976; Köller et al. 1999; Szkolnik and Gilpatrick 1969). Sensitivity testing on a discriminatory concentration of 3.0 µg/ml dodine revealed that 12% of samples collected from fungicide treated orchards in 2008 had reduced sensitivity (Seyran et al. 2010a). The frequency of insensitive samples was low in 2014 with roughly 55% of samples testing completely sensitive but levels of insensitivity were observed in 72% of samples tested in 2015, indicating that a shift to insensitivity is occurring slowly over time (K. L. Stevenson, unpublished).

The phosphonates are considered to have a low risk of resistance (FRAC 2016a). However, insensitivity to fosetyl-aluminum has been observed in a few species of oomycetes, including isolates of *Bremia lactucae* Regal collected across lettuce production fields in California (Brown et al. 2004). Additionally, isolates of *Phytophthora cinnamomi* Rands have been shown to vary in sensitivity to phosphorus acid (Wilkinson et al. 2001). Reduced sensitivity to these chemicals has not been observed in isolates of *V. effusa* to date.

The demethylation inhibitor fungicides (DMIs) are widely used for scab management and are thought to have a medium risk for resistance developing (FRAC 2016a). Monoconidial isolates of *V. effusa* collected in 2003 were significantly less sensitive to a discriminatory concentration of 0.2 µg/ml propiconazole when compared with isolates from 1995 (Stevenson et al. 2004; Reynolds et al. 1997). Additionally, substantial reductions in sensitivity were observed in 63% orchards samples, tested at the discriminatory concentration of 1.0 µg/ml propiconazole, and compared with baseline

samples in 2008 (Seyran et al. 2010a). Levels of insensitivity rose to 77 and 86% in orchard samples collected in 2014 and 2015 (K. L. Stevenson, unpublished). Practical resistance to the DMIs has been observed in 17 fungal species and tends to develop as a result of target site mutations in the *cyp51* gene, overexpression of *cyp51*, or reduced intracellular fungicide accumulation due to the activity of efflux transporters (Price et al. 2015; Ziogas and Malandrakis 2015; FRAC 2016a).

Similar to the mechanisms of resistance to other fungicides, point mutations in the *cyp51* gene that lead to amino acid substitutions are the most frequently observed cause of resistance (Price et al. 2015). More than 30 different amino acid substitutions have been found to lower the fungicide's affinity for binding to *cyp51* resulting in varying levels of DMI sensitivity (Price et al. 2015). Notable substitutions include phenylalanine for tyrosine at position 136 or the equivalent residues at positions 134 or 137 (Y136F or Y134F/Y137F); glycine for alanine at position 379 (A379G); and valine for isoleucine at position 381 (I381V); among several others (Ziogas and Malandrakis 2015; FRAC 2016a). Additionally, several substitutions occurring at different positions at the same time may combine to cause varying amounts of resistance to different DMIs (Price et al. 2015). However, resistance is not always associated with a target site mutation; in some cases, resistance was observed as a result of an overexpressed *cyp51* gene (Ma et al. 2006; Schnabel and Jones 2001). This overexpression is caused by alterations in the promoter region of *cyp51* by the insertion of tandem repeats or transposable elements (Price et al. 2015). An increase in mRNA levels is thought to correlate with an increase in cellular *cyp51* levels, thus more targets for the constant amount of fungicide are present, which results in DMI insensitivity (Price et al. 2015). The third mechanism has

been more widely reported in fungal pathogens of humans but has occurred in a limited number of fungal plant pathogens (Price et al. 2015). Membrane-bound drug transporters are able to secrete antifungal compounds to the extracellular space of mycelial cells, preventing the intracellular accumulation of the fungicide at its target site (Ziogas and Malandrakis 2015). Two important classes of transporter proteins that can play a major role in fungicide sensitivity are the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters (Del Sorbo et al. 2000). Cross resistance between members of this group is generally accepted to occur between fungicides used to manage the same pathogens (FRAC 2016a).

The QoI fungicides are classified as "high-risk" for resistance to develop in fungal populations due to their single-site mode of action (FRAC 2016a). Practical resistance to the QoIs has been documented in over 50 fungal species representing 35 genera (FRAC 2018) and primarily occurs as a result of nucleotide point mutations in the *cyt b* gene (Fernández-Ortuño et al. 2008). These mutations in the *cyt b* gene lead to amino acid substitutions within the region corresponding to amino acid residues 120 to 155 of the encoded protein. Substitutions at three positions within this specific region have been detected in QoI-resistant plant pathogenic fungi and oomycetes. A substitution of alanine for glycine at position 143 (G143A) is confers complete resistance to the QoIs and is associated with a failure to inhibit the pathogen (Fernández-Ortuño et al. 2008). Substitutions of leucine for phenylalanine at position 129 (F129L) or arginine for glycine at position 137 (G137R) have been associated with reduced sensitivity, but not complete resistance; the pathogen usually can be managed by more frequent applications or increased rates of QoIs (Fernández-Ortuño et al. 2008; Gisi et al. 2007; Sierotzki et al. 2007). Due to their common mode of action, cross-resistance within this class of chemicals is frequently

observed in fungi carrying amino acid substitutions, so that if resistant to one QoI active ingredient, the fungus will be resistant to all QoI active ingredients (Fernández-Ortuño et al. 2008; Sierotzki et al. 2000). To date, practical resistance has not been reported in *V. effusa* even with the "high-risk" classification and repeated use of fungicides from this group in the same orchards over several years. However, there are unconfirmed reports of decreased efficacy in some locations across southwest Georgia (T. B. Brenneman, personal communication).

Another potential mechanism of QoI resistance occurs due to an alternative cyanide-resistant respiration pathway, sustained by alternative oxidase (AOX) that has been shown to serve as a rescue mechanism for some plant pathogens in the presence of a QoI fungicide (Fernández-Ortuño et al. 2008; Wood and Hollomon 2003). When AOX occurs, mitochondrial electron transfer is diverted by circumventing the cytochrome bc_1 complex; the target site of the QoIs. This alternative respiration pathway is thought to provide enough energy to counteract the inhibitory effects of QoIs in vitro but not in planta (Fernández-Ortuño et al. 2008). Salicylhydroxamic acid (SHAM) and propyl gallate (PG) are characteristic inhibitors of AOX in vitro (Wood and Hollomon 2003). To prevent the fungus from utilizing this alternative respiration pathway and circumventing normal electron transfer, SHAM or PG are often added to a medium to conduct in vitro bioassays for QoIs. Seyran et al. (2010b) found that both of these AOX inhibitors exhibit fungitoxic effects on *V. effusa* in vitro and cannot be included when estimating QoI sensitivity. Due to the inability of an in vitro bioassay (without SHAM or PG) to accurately quantify sensitivity to the QoIs, molecular methods may provide a better means of monitoring the effectiveness of this important class of fungicide.

An additional factor impacting QoI resistance development is the potential presence of an intron directly downstream of position 143. This intron prevents the formation of the G143A substitution in some plant-pathogenic fungi, presumably decreasing the risk for resistance development, and has been observed in *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., *Monilinia fructicola* (Wint.) Honey, *M. laxa* (Aderh. & Ruhland) Honey, *Phyllosticta ampelicida* (Engelm.) Aa, *Pyrenophora teres* (Drechsler), and several species of rust fungi (Banno et al. 2009; Grasso et al. 2006a; Grasso et al. 2006b; Luo et al. 2010; Miessner and Stammler 2010; Miessner et al. 2011; Sierotzki et al. 2007).

Stability of resistance and fitness

The development and phenotypic stability of resistance are largely dependent on the fitness of the resistant members of a population (Cox et al. 2007; Ishii 2015). Fitness can be defined as the survival and ability of an organism to reproduce successfully (Pringle and Taylor 2002). With the assumption that some level of underlying variation in sensitivity exists, the use of fungicides for disease management will result in selection on a plant pathogen population. In such an environment, resistant isolates have a clear fitness advantage over sensitive isolates and will be selected for, leading to an increase in the frequency of resistant isolates in the pathogen population over subsequent generations (Peever and Milgroom 1994). However, in the absence of the fungicide, resistant isolates may have lower fitness due to mutations associated with resistance that may potentially disrupt important physiological or biochemical processes (Zhan and McDonald 2013). Stability of resistance occurs when a certain level of fungicide insensitivity is preserved in a pathogen after successive generations of exposure or no exposure to the target fungicide (Ishii 2015). If resistant isolates are less fit than those sensitive to the fungicide, the frequency of resistant isolates in the population will decrease in the

absence of the fungicide, and resistance will not be stable. The cost of resistance may affect the ability of the fungus to complete its life cycle and may specifically affect conidia production and dispersal, infection efficiency, mycelial growth, overwintering capabilities, and ability to compete with other strains in a field environment, among other life cycle components (Mikaberidze and McDonald 2015). The portion of the pathogen population consisting of resistant isolates with reduced fitness will decline in the absence of fungicide selection pressure, which, over time, could result in a population that is mostly sensitive to the fungicide, rendering it effective again (Ishii 2015). For example, during a competition study with a mixture of an azoxystrobin-resistant and –sensitive isolate of *Magnaporthe oryzae* B. C. Couch on perennial ryegrass (*Lolium perenne* L.), the sensitive isolate produced significantly more conidia and increased in frequency over time in the absence of azoxystrobin (Ma and Uddin 2009). Likewise, isolates of the sugar beet leaf spot pathogen, *C. beticola*, resistant to the organotin fungicides, fentin acetate and fentin chloride, were found to produce less severe leaf spot epidemics on untreated sugar beets compared with sensitive isolates (Giannopolitis and Chrysayi-Tokousbalides 1980). In 1994, TPTH resistant isolates of *C. beticola* were first reported in the U.S. and a subsequent 10-year fungicide sensitivity monitoring program revealed a decrease in frequency of resistant isolates over time when annual TPTH applications were reduced (Bugbee 1995; Secor et al. 2010). Furthermore, the frequency of DMI-resistant isolates of *C. beticola* decreased significantly during an epidemic in competition with DMI-sensitive isolates (Karaoglanidis et al. 2001). Interestingly, the instability of propiconazole resistance was observed in isolates of *Monilinia fructicola* as percent inhibition increased significantly following both consecutive transfers in vitro and

months in cold storage (Cox et al. 2007). In contrast, no fitness costs were identified in QoI-resistant isolates of *Alternaria alternata* (Fr.) Keissl and resistance was found to be stable after multiple generations (Vega and Dewdney 2014; Karaoglanidis et al. 2011). Similarly, MBC resistance has been stable for several decades with resistant and sensitive isolates appearing equally fit (Jones and Ehret 1976). Resistant isolates of *C. beticola* were identified decades after MBC use ceased (Campbell et al. 1998). Conversely, in some instances the frequency of MBC-resistant isolates has decreased in the absence of the fungicide, only to reappear rapidly once that fungicide was used again (McGrath 2001). However, reduced fitness in fungicide-resistant isolates appears to be dependent on the pathogen and potentially on the mode of action of the fungicide (Hollomon 2015b).

Justification and objectives

The most practical method for managing scab is to make multiple preventive fungicide applications throughout the growing season (Brock and Bertrand 2007a). These applications are typically made on a calendar schedule, but the interval between sprays is highly dependent on weather, which may lead to many more applications in wet years compared to dry years (Latham 1995). *Venturia effusa* has a high reproductive capacity, which leads to rapid population growth and an increased opportunity for the selection of fungicide resistance (FRAC 2013a). Isolates of *V. effusa* resistant to the MBC active ingredient benomyl were first reported in 1975 after just three years of effective control; insensitivity to MBC thiophanate-methyl remains an issue today (Littrell 1976; Stevenson et al. 2015). Additionally, a significant reduction in sensitivity to DMI active ingredients was detected when comparing monoconidial isolates from

1995 to those collected in 2003 (Stevenson et al. 2004). In 2008, 2014, and 2015, reduced sensitivities to dodine and triphenyltin hydroxide were documented in the Georgia population of *V. effusa* (Seyran et al. 2010a; Stevenson et al. 2015). The QoI fungicides have been used on pecan since the late 1990s but practical resistance has never been reported. Efforts to monitor QoI sensitivity have been hindered by the inability of in vitro methods to accurately quantify sensitivity to this important chemical group.

Scab control is often the greatest operating cost for commercial growers and the studies conducted as part of this dissertation were directed to better understand the biology of fungicide-resistant *V. effusa* to improve the effectiveness of fungicide applications in Georgia. The specific objectives were to (1) investigate the potential for quinone outside inhibitor (QoI) fungicide resistance in *V. effusa* (Chapters 2 and 3), (2) establish the relationship between fungicide efficacy and fungicide sensitivity (Chapter 4), (3) determine appropriate sampling strategies for accuracy in monitoring sensitivity of *V. effusa* to multiple fungicides (Chapter 5), and (4) explore fitness and stability of insensitivity to fentin hydroxide and tebuconazole in isolates of *V. effusa* (Chapter 6).

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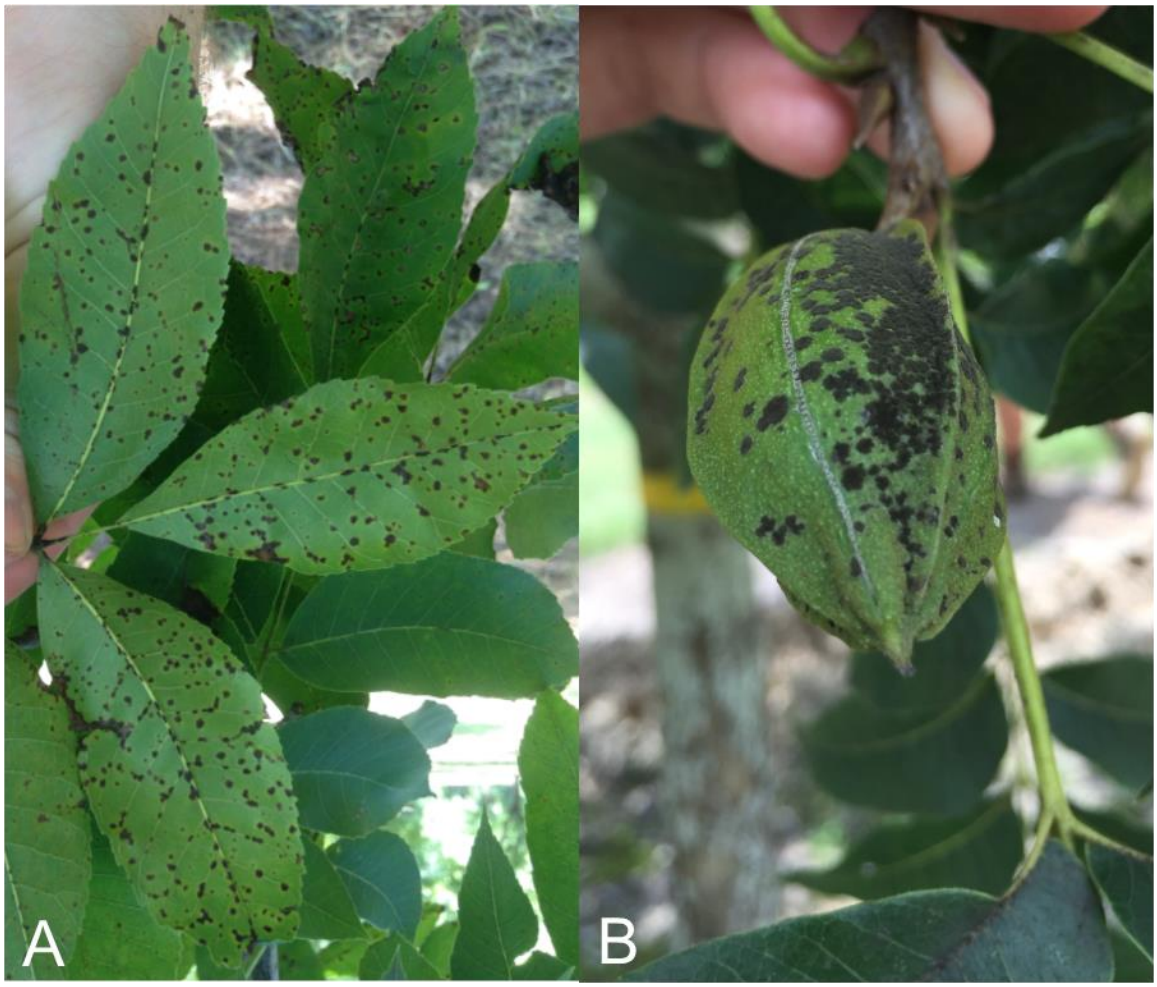

Figure 1.1. Symptoms of pecan scab. **A.** Small olive-brown to black lesions typical of pecan scab observed on the abaxial leaf surface. **B.** Small, circular lesions on a scab infected nut.

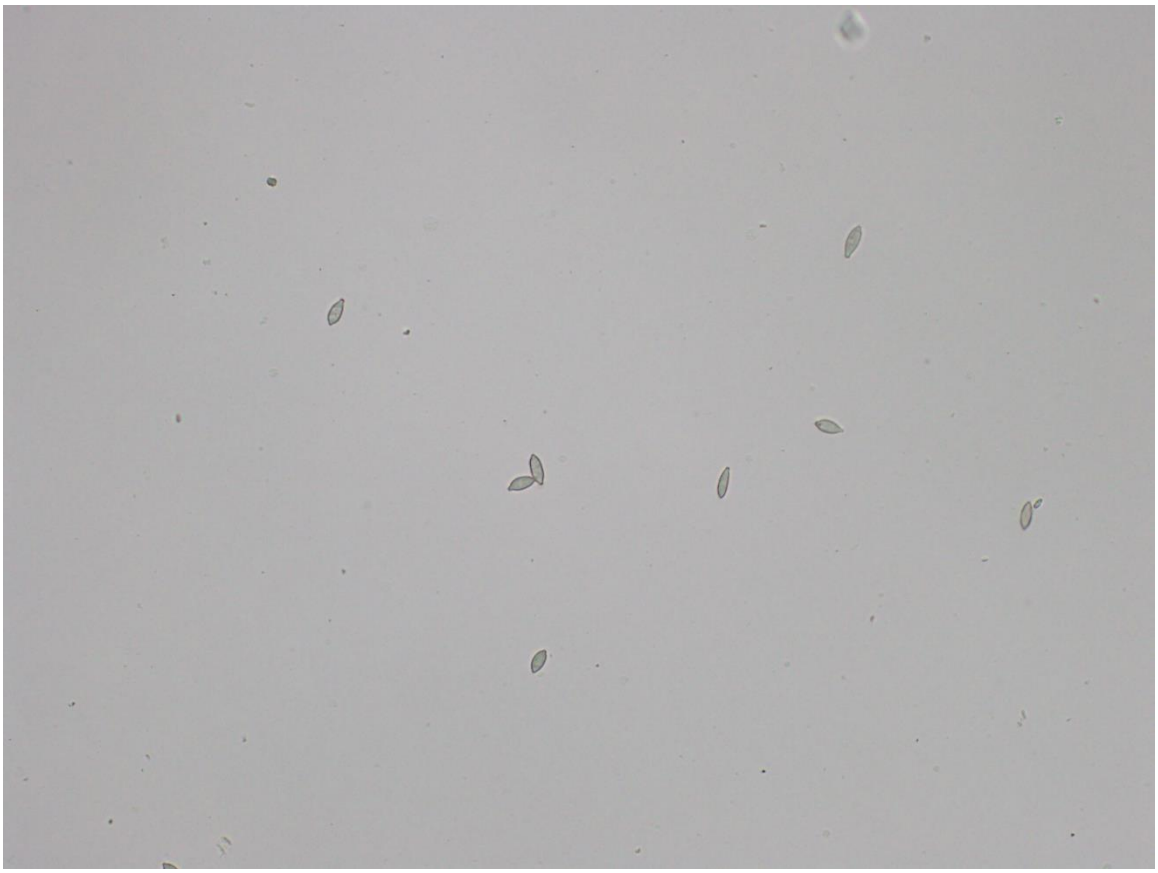

Figure 1.2. Conidia of *Venturia effusa* observed at a magnificantion of 200×

CHAPTER 2

LOCATION OF AN INTRON IN THE CYTOCHROME *B* GENE INDICATES REDUCED RISK OF QOI FUNGICIDE RESISTANCE IN *FUSICLADIUM EFFUSUM*[1]

[1]Standish, J. R., Avenot, H. F., Brenneman, T. B., and Stevenson, K. L. 2016. Accepted by *Plant Disease*. Reprinted here with permission of the publisher, The American Phytopathological Society.

Abstract

Pecan scab, caused by *Fusicladium effusum* (Wint.), is most effectively managed using multiple fungicide applications including quinone outside inhibitors (QoIs). However, QoIs have a high risk for resistance developing in phytopathogenic fungi. QoI resistance is generally associated with amino acid substitutions at positions 129, 137, and 143 of the cytochrome *b* (cyt *b*) gene. A substitution at position 143 confers complete resistance, while an intron immediately downstream of this position prevents the substitution. The objective of this study was to assess the risk of QoI resistance by characterizing a partial fragment of the *F. effusum* cyt *b*. Sequence analysis of the 1,919-bp fragment revealed the presence of a 1,407-bp intron immediately downstream of position 143. This intron was identified in 125 isolates collected from 16 counties across the state of Georgia. No substitutions were identified at positions 129 or 143, but in seven of the isolates, glycine was replaced with serine at position 137. The ubiquitous nature of the detected intron provided strong evidence that the G143A substitution may not occur in *F. effusum* isolates, although resistance could still develop through intron loss events, the selection of intron-lacking genotypes, or as the result of other mutations in the cyt *b* gene.

Introduction

Pecan scab, caused by the fungus *Fusicladium effusum* (Wint.), is the most destructive disease affecting pecan [*Carya illinoinensis* (Wangenh.) K. Koch] (Demaree 1924). *Fusicladium effusum* is a slow-growing fungus and a member of the family Venturiaceae with an unknown sexual stage (Beck et al. 2005; Seyran et al. 2010a). Scab occurs as lesions on leaves, fruit shucks, and twigs. Young, actively growing tissues are most susceptible to infection and become resistant to infection as they mature (Demaree 1924; Littrell and Bertrand 1981). Small olive-brown to black spots of approximately 1 to 5 mm in diameter will appear on infected plant material, and these spots may expand and coalesce to form large, irregularly shaped lesions. Sporulating lesions have a velvety or rough appearance (Bertrand 2002; Demaree 1924). Shuck infections that occur between fruit set and shell hardening can cause severe yield loss. Fruit infections that occur during the early stages of fruit development can cause premature fruit drop; although infections that occur after the shell has hardened are thought to be more cosmetic than damaging (Bertrand 2002; Demaree 1924; Hunter 1983).

The use of resistant cultivars is the most practical method of managing scab. However, host resistance is not permanent as cultivar-specific physiological races of *F. effusum* are known to exist, and over the years have developed on cultivars once thought to be highly scab resistant (Demaree and Cole 1929; Sparks 1992). In the absence of varietal resistance, the most effective scab management practice is to make multiple preventive fungicide applications throughout the growing season (Brock and Bertrand 2007a). Fungicides are typically sprayed on a calendar schedule, but the interval between sprays is often adjusted based on weather conditions (Latham 1995). The general

guideline in Georgia calls for 7 to 10 fungicide applications per season, made on 10-14 day intervals from bud break until pollination, and on 14-21 day intervals from pollination to shell hardening (Brock and Bertrand 2007b). During rainy periods, fungicides may be applied every 7 to 10 days, which can substantially increase the total number of applications per season. Fungicides from different chemical groups are approved for use on pecan in the U.S. and include the quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs), methyl benzimidazole carbamates (MBCs), guanidines, and organotin compounds (Fungicide Resistance Action Committee [FRAC] Code 11, 3, 1, U12, and 30, respectively) (Brock et al. 2007).

The QoI fungicides are a major tool in scab management both as premix combinations, most often with a DMI, and as stand-alone products. Several stand-alone QoI fungicides are commercially available and labeled for use on pecan in the U.S., most notably azoxystrobin (as Abound 2.08F; Syngenta Crop Protection, Greensboro, NC), kresoxim-methyl (as Sovran 50WG; BASF Corporation, Research Triangle Park, NC), and pyraclostrobin (as Headline 2.09F; BASF Corporation, Research Triangle Park, NC) (Brock et al. 2007). The fungicide resistance action committee (FRAC) classifies the QoIs as being "high-risk" for developing resistance in fungal populations due to their single-site mode of action (FRAC 2016). The fungicides in this group inhibit mitochondrial respiration by binding to the quinol oxidation site of the cytochrome bc_1 enzyme complex, blocking electron transfer between the cytochrome *b* (cyt *b*) and cytochrome c_1 (Bartlett et al. 2002; Gisi et al. 2002). This process halts the production of ATP resulting in an energy deficiency in fungal cells (Fernández-Ortuño et al. 2008).

Resistance to the QoI fungicides was first detected in isolates of *Blumeria graminis* (DC) EO Speer f. sp. *tritici* identified in 1998 in Germany (Bartlett et al. 2002). Since that time, field resistance has been documented in over 30 species representing 20 genera (FRAC 2013) and primarily occurs as a result of nucleotide point mutations in the cyt *b* gene (Fernández-Ortuño et al. 2008). These mutations lead to specific amino acid substitutions and have been detected in an area of the cyt *b* gene corresponding to amino acid positions 120 to 155 of the encoded protein. Within this specific region, three substitutions have been detected in QoI-resistant plant pathogenic fungi and oomycetes. A substitution from glycine to alanine at position 143 (G143A) is known to confer complete resistance to the QoI fungicides and is associated with a failure to inhibit the pathogen (Fernández-Ortuño et al. 2008). Additionally, substitutions from phenylalanine to leucine at position 129 (F129L) or from glycine to arginine at position 137 (G137R) have been associated with reduced sensitivity; but the pathogen usually can be managed by more frequent applications or increased rates of the QoI fungicides (Fernández-Ortuño et al. 2008; Gisi et al. 2002; Sierotzki et al. 2007). Due to their common mode of action, cross-resistance within this class of chemicals is frequently observed in fungi carrying amino acid substitutions, so that if resistant to one QoI active ingredient, the fungus will be resistant to all QoI active ingredients (Fernández-Ortuño et al. 2008; Sierotzki et al. 2000).

An alternative cyanide-resistant respiration pathway sustained by alternative oxidase (AOX) has been shown to serve as a rescue mechanism for some plant pathogens in the presence of a QoI fungicide (Fernández-Ortuño et al. 2008; Wood and Hollomon 2003). When AOX occurs, mitochondrial electron transfer is diverted by circumventing

the inhibitory site of the QoIs. This alternative pathway seems to provide enough energy to counteract the effects of QoIs in vitro but not in planta (Wood and Hollomon 2003). Characteristic inhibitors of AOX in vitro include salicylhydroxamic acid (SHAM) and propyl gallate (PG) (Schonbaum et al. 1971; Siedow and Bickett 1981).

In 2014, a fungicide sensitivity monitoring program was initiated in Georgia that allowed pecan growers to submit leaf samples exhibiting symptoms of pecan scab for orchard-specific testing. Sensitivities to dodine, fentin hydroxide, thiophanate-methyl, propiconazole, and tebuconazole were determined using a rapid in vitro method described by Seyran et al. (2010b). Samples were also tested for sensitivity to azoxystrobin; however, SHAM and PG have been shown to exhibit fungitoxic effects on *F. effusum* in vitro, making the results of such a test unreliable (Seyran et al. 2010c). Due to the inability of an in vitro bioassay to accurately quantify sensitivity to the QoIs, coupled with the very slow growth rate of the pathogen in culture, a molecular method may provide a more practical and accurate means of monitoring the effectiveness of this important class of fungicide. Additionally, the presence of an intron immediately downstream of position 143 has been found to prevent the formation of the G143A substitution in plant pathogenic fungi, presumably mitigating the risk of resistance (Sierotzki et al. 2007; Grasso et al. 2006; Luo et al. 2010).

The objectives of this study were to sequence and characterize a partial fragment of the *F. effusum* cyt *b* gene and assess the risk of QoI-resistance developing by i) screening for the amino acid substitutions known to confer resistance to the QoI fungicides; and ii) determining the presence or absence of an intron immediately downstream of position 143 in isolates collected from pecan orchards in Georgia.

Materials and Methods

Collection of *F. effusum* isolates. Samples of pecan leaflets showing symptoms of scab were collected by individual growers and submitted to the University of Georgia Coastal Plain Experiment Station in Tifton, GA. Each sample was divided into three groups of 15 leaflets and conidia were collected using a protocol described by Seyran et al (2010b). To confirm pathogen identity, leaf lesions were examined using a stereomicroscope to identify the conidia of *F. effusum*. Species confirmation was initially based on conidial morphology using light microscopy (×100); additionally, isolates were positively identified after sequencing and analysis of the internal transcribed spacer (ITS) region (Bertrand 2002; White et al. 1990).

Monoconidial isolates were established from each group per sample by pipetting 19 µL of the corresponding spore suspension onto water agar (15 g agar per liter of water) amended with tetracycline (50 mg/liter), streptomycin sulfate (50 mg/liter), and chloramphenicol (50 mg/liter) in petri plates (15 × 100 mm diameter). The spore suspension was then spread across the surface with a sterile glass rod. Two replicate plates were prepared per sample. After 24 h, three germinated conidia per sample were aseptically transferred to petri plates containing potato dextrose agar (PDA) made with the previously stated antibiotic amendments. Monoconidial isolates were placed in an incubator at 25ºC to allow for colony growth.

Genomic DNA and RNA isolations and cDNA synthesis. Genomic DNA was extracted from mycelia for 125 *F. effusum* isolates obtained from infected samples collected in 2014. Isolates were grown on agar medium as previously described; mycelia

and conidia were collected after 6 to 8 weeks incubation by scraping the colony surface with a sterile blade. Genomic DNA was extracted and purified using a ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's instructions.

For RNA isolation, mycelia for six *F. effusum* isolates were grown in 100 mL of potato dextrose broth (PDB) for 4 days at 24°C. Mycelia were removed from the broth, rinsed with sterile distilled water, and dried before being ground to a fine power in liquid nitrogen using a mortar and pestle. Total RNA was extracted from mycelia using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA samples were subsequently treated with DNase I Amp Grade (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was then synthesized from 5 µg of total RNA using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Cloning of a partial cytochrome *b* gene fragment from *F. effusum* isolates A degenerate primer set was designed based on highly conserved regions of cyt *b* gene sequences from several fungi, namely *Venturia inaequalis* (Cooke) G. Winter (Genbank Acc. No. AF004559), *Cercospora beticola* Sacc. (Genbank Acc. No. JQ360628), *C. kikuchii* (Tak. Matsumoto & Tomoy.) (Genbank Acc. No. AB231863), *Mycosphaerella graminicola* (Fuckel) J. Schröt (Genbank Acc. No. AY247413), and *Alternaria alternata* (Fr.) Keissl (Genbank Acc. No. DQ209283). These primers, PecFwd (5' – GAGGKYTATATTAYGGTTCTTAYAGAG – 3') and PecRev (5' – GAGTTTGCATAGGGTTAGCTA – 3') were used to amplify a ~450 bp partial

fragment of the cyt *b* gene from the first-strand cDNA in *F. effusum*. ThermoPrime DNA polymerase (Invitrogen, Carlsbad, CA) was used in PCR reactions heated at 95°C for 3 min, followed by 40 cycles of amplification (94°C for 15 s, 50°C for 30 s, and 72°C for 30 s), and a final extension at 72°C for 5 min. The PCR products for the six *F. effusum* isolates were visualized by electrophoresis in 1.0% agarose gels and purified using a Geneclean kit (MP Biomedicals, Santa Ana, CA). The purified products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced in both directions at the University of Georgia Genomics Facility. The nucleotide sequences for two isolates, 4_PeS and 10_PeS, were deposited into GenBank (Acc. No. KT427434 and KT427435, respectively).

Molecular characterization of *F. effusum* isolates. Isolates were positively identified based on nucleotide sequences of the internal transcribed spacer (ITS) region of three representative *F. effusum* isolates: 14-49, 14-73, 14-174. Polymerase chain reaction (PCR) amplification was performed using an ITS4/ITS5 primer set (White et al. 1990).

The primers PS1 (5’ – GTTACAGCCTTCCTGGGTTAT – 3’) and PR1 (5’ - AGGCCTCCCCACAGAAATTCG – 3’), described by Fontaine et al. (2009) for use with the closely related fungus *V. inaequalis*, were used to amplify a partial fragment of the cyt *b* gene corresponding to the binding site of the QoI fungicides. Using the resultant sequences and the cDNA sequences previously described, primer sets FeCytb_F1 (5’ - GAGCACCTAGAACGTTAGTGTGA – 3’) and FeCytb_R1 (5’ - AACCAGACGGGCTCTAATGG – 3’); FeCytb_F2 (5’ - TGTTTGTTGGTCTAGTAGATGGGG – 3’) and FeCytb_R2 (5’ - GCTAACCAAGACGCACCTGTA – 3’); and FeCyb_F3

(5’ - AGGTAACTTCGGACAATGTACTG – 3’) and FeCytb_R3 (5’ - TCACGCTGAAACCTCCTCAC – 3’) were designed to amplify a region that would allow for the detection of nucleotide point mutations at positions 129 (F129L), 137 (G137R), and 143 (G143A). These same primers allowed for the identification of introns within the partial gene fragment.

PCR was carried out in a Mastercycler Thermal Cycler (Eppendorf AG, Hamburg, Germany) with 1× GoTaq Buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of forward and reverse primer, 1 unit GoTaq polymerase, and 20 to 80 ng/µL genomic DNA in a final reaction volume of 25 µL. The cycling conditions for all primer sets were an initial denaturation period at 94ºC for 3 min; 35 cycles of amplification (94ºC for 30 s, 55ºC for 45 s, and 72ºC for 50 s), and a final extension at 72ºC for 10 min. PCR products were visualized by electrophoresis in 1.5% agarose gels and purified using a QiaQuick PCR Purification Kit (Quiagen Inc., Valencia, CA) per the manufacturer’s instructions. Amplicons were submitted to Eurofins MWG Operon LLC, Louisville, KY for custom sequencing. Nucleotide sequences were trimmed and contigs were assembled using the BioEdit software package version 7.2.5 (Hall 1999). Alignment and analysis were performed using ClustalW within MEGA6 (Tamura et al. 2013).

Results

Collection of *F. effusum* isolates and genomic DNA extraction. A total of 125 monoconidial isolates were obtained from samples submitted from orchards in 16

different counties across the state of Georgia (Table 2.1). Genomic DNA was extracted successfully from all isolates collected for this study.

Molecular characterization of *Fusicladium effusum* isolates. The nucleotide sequences of the ITS region of three representative isolates (14-49, 14-73, and 14-174) were mutually identical and shared 100% homology to the corresponding genomic region of *F. effusum* strain STE-U 4525 (GenBank Acc. No. AY251085). The nucleotide sequences of 14-49, 14-73, and 14-174 were deposited into GenBank (Acc. No. KT387749 – KT387751).

Primers PS1/PR1 successfully amplified a single DNA band of 1,840 bp, which upon sequencing, shared 99% homology with a 409-bp portion of the corresponding region in *V. inaequalis* (GenBank Acc. No. AF004559). This primer set was expected to amplify a region of approximately 400 bp and because the resulting amplicon was >1,800 bp, three new primer sets were designed to amplify the larger fragment in three overlapping sections. Primer pairs FeCytb_F1/FeCytb_R1; FeCytb_F2/FeCytb_R2; and FeCytb_F3/FeCytb_R3 successfully amplified three single DNA bands (excluding primer regions) of 619, 883, and 508 bp, respectively. These overlapping fragments were combined to form consensus sequences that were analyzed for twenty individual isolates. The resulting fragment of 1,919 bp (excluding primer regions), spanning amino acid positions 115 – 164, revealed the presence of two introns (Fig. 2.1A).

The first intron (360 bp) was observed immediately downstream of the codon for proline at position 135 and shared 99% homology with an intron of the same size found at the same position in *V. inaequalis* (GenBank Acc. No. AF004559). A short exon (24 bp) separated the first intron from a second intron (1,407 bp) that was identified directly

downstream of the codon for glycine at position 143. A BLAST search of the predicted amino acid sequence of this 1,407-bp intron in the Non-Redundant Protein Sequence Database (nr) revealed the presence of a putative conserved domain belonging to the LAGLIDADG endonuclease superfamily; but high homology (>70%) was not identified and only a few minor similarities were noted (>40%-<70%). The nucleotide sequences of the entire 1,919-bp fragment encompassing both introns for isolates 14-17, 14-28, 14-49, 14-88, and 14-177 were deposited into GenBank (Acc. No. KT387752 – KT387756).

The primers FeCytb_F1/FeCytb_R1 were designed with the reverse primer beginning 192 bp inside of the intron downstream of position 143. As such, this primer set amplified a band of 619 bp which allowed for the identification of mutations in the remaining 105 *F. effusum* isolates, in addition to positively confirming the presence of both introns in all isolates prior to sequencing. Sequence analysis of these PCR products revealed the presence of triplets coding for phenylalanine (F) at position 129, and glycine (G) at position 143. However, in seven isolates, a single nucleotide mutation (G➔A) was identified in the codon corresponding to position 137 of the cyt *b* gene (GGT➔AGT). This mutation resulted in an amino acid substitution from glycine to serine (G137S) (Fig. 2.1B). No other differences in nucleotide sequences resulting in amino acid substitutions, specifically F129L and G143A, were observed in isolates of *F. effusum* used in this study.

Partial nucleotide sequences of the cyt *b* gene for the seven *F. effusum* isolates carrying a G137S substitution (14-33, 14-68, 14-70, 14-92, 14-95, 14-103, and 14-133) and seven wild-type *F. effusum* isolates (14-1, 14-19, 14-40, 14-61, 14-81, 14-86, and 14-

115) studied in this work were deposited into GenBank (Acc. No. KT387757 – KT387770).

Discussion

The standard pecan scab management protocol is based on the use of multiple preventive fungicide applications (Demaree and Cole 1929). The fungicides labelled for pecan belong to different chemical groups that include the QoIs, DMIs, MBCs, guanidines, and organotin compounds (Brock et al. 2007). The inherent risk of resistance developing in *F. effusum* to the active ingredients within each of these groups ranges from "low to medium" to "high" (FRAC 2016), which makes screening isolates to determine their sensitivity profiles of the utmost importance. Isolates of *F. effusum* resistant to the MBC active ingredient benomyl were first reported in 1975 after just three years of effective control (Littrell 1976). Additionally, a significant reduction in sensitivity to DMI active ingredients was detected when comparing monoconidial isolates from 1995 to those collected in 2003 (Stevenson et al. 2004). To date, even though QoIs are considered "high-risk" for resistance development and have been used on pecans for several years, practical resistance to these fungicides has not been reported. Additionally, shifts in fungicide sensitivity have not been identified in isolates of *F. effusum*, although a reliable screening method does not currently exist.

The initial focus of this study was to determine whether point mutations associated with QoI resistance in other fungi were present in a partial fragment of the *F. effusum* cyt *b* gene. The investigation concentrated on an area of the gene corresponding to amino acid residues 115 to 164. Within this region, several nucleotide mutations

leading to amino acid substitutions can occur that are known to confer resistance to the QoI fungicides, although the level of resistance varies with each substitution. The most important change, occurring at position 143 (G143A), is known to confer complete resistance, while a substitution at position 129 (F129L) or 137 (G137R) has been associated with moderate or partial resistance (Fernández-Ortuño et al. 2008; Gisi et al. 2002); Sierotzki et al 2007). In this study, nucleotide mutations resulting in the G143A and F129L substitutions were not identified; however, a polymorphism in the first position of the codon corresponding to residue 137 (G➔A) resulting in an amino acid substitution from glycine to serine (GGT➔AGT) was identified in seven of the 125 *F. effusum* isolates screened. To our knowledge, this is the first report of a substitution from glycine to serine at this position in a phytopathogenic fungus. Sierotzki et al. (2007) reported that isolates of *Pyrenophora tritici-repentis* (Died.) Dreschsler carrying a substitution at position 137 (G137R) exhibited decreased sensitivity when compared to the wild-type isolates, but this insensitivity was considered to be a form of partial resistance similar to that caused by the F129L substitution also reported in that work. The mutation leading to the amino acid substitution occurred at the first nucleotide of codon 137 in isolates of *P. tritici-repentis* and the isolates of *F. effusum* used in this study (Sierotzki et al. 2007). Thus it would appear that with a shift from G➔A in the first position of the codon, the potential for other amino acids to replace glycine would be specific to the triplet coding for glycine in that gene, within a particular species. Nucleotide shifts resulting in different amino acid substitutions at position 137, such as G137V and G137E, in non-phytopathogenic organisms were found to reduce the respiratory capacity of the organism, indicating that there may be a potential fitness

penalty associated with a mutation at this position (Brasseur et al. 1996; Fisher and Meunier 2001). Without a reliable in vitro method to identify phenotypic characteristics, the G137S substitution could not be properly characterized in the *F. effusum* isolates used in this work. Therefore, future studies are necessary to characterize the isolates and determine the relevance of this substitution in *F. effusum*.

A major finding of this research was the presence of a 1,407-bp intron just downstream of the codon for glycine at position 143 in all 125 isolates of *F. effusum* examined in this study. This provided strong evidence that the G143A amino acid substitution may not occur in isolates of *F. effusum,* as this substitution has only been reported in pathogens not carrying the intron at this position (Grasso et al. 2006; Siertozki et al. 2007). An intron adjacent to position 143 has been observed in *Alternaria solani* Sorauer, *Botrytis cinerea* Pers., *Monilinia fructicola* (Wint.) Honey, *M. laxa* (Aderh. & Ruhland) Honey, *Phyllosticta ampelicida* (Engelm.) Aa, *Pyrenophora teres* (Dreschsler), and several species of rust fungi (Banno et al 2009; Grasso et al. 2006a; Grasso et al. 2006b; Luo et al. 2010; Miessner and Stammler 2010; Miessner et al. 2011; Sierotzki et al 2007). The intronic sequence downstream of position 143 in isolates of *F. effusum* belongs to the group I intron family (Cech 1988; Lambowitz and Belfort 1993). As has been reported in other organisms, the exonic base immediately upstream of the 5' splice site was always a T (U in pre-mRNA) and the base that followed the 3' splice site was always a G (Banno et al. 2009; Cech 1988; Grasso et al. 2006a; Luo et al. 2010; Sierotzki et al. 2007). Additionally, a BLAST search of the intron sequence revealed the presence of a putative conserved domain belonging to the LAGLIDADG endonuclease superfamily, which has been associated with group I introns (Belfort and Roberts 1997;

Heath et al. 1997). Group I introns are able to form an active site to mediate their own removal from mRNA transcripts (Cech 1988). Because splice-site recognition relies on internal guide sequences of the intron that pair with exon sequences, a mutation at the splice site will hinder pairing and the correct excision of the intron (Vallières et al. 2011). Therefore, a nucleotide mutation at a position near an exon/intron junction, such as that which leads to the G143A substitution, would affect the splicing process and lead to a deficient cyt *b* (Cech 1988; Lambowitz and Belfort 1993; Vallières et al. 2011). Mutant isolates carrying the G143A substitution and the aforementioned intron could certainly exist at some point in time; although, because of the deficient cyt *b*, these isolates would likely exhibit reduced fitness and quickly disappear from the population (Vallières et al. 2011).

The cyt *b* gene has been reported to be highly conserved in a single species with regards to the placement of this intron; to date, there has been only one observed exception (Banno et al 2009; Grasso et al. 2006a; Grasso et al. 2006b; Luo et al. 2010; Miessner and Stammler 2010; Miessner et al. 2011; Sierotzki et al 2007). Banno et al. (2009) identified isolates of *B. cinerea* from Japan that had two distinct cyt *b* profiles; the first had a group I intron (1,205 bp) downstream of position 143, while the second profile did not possess the intron. Isolates without the intron developed the G143A substitution and consequently were found to be highly resistant to the QoI fungicides. Divergent cyt *b* genotypes could exist in populations of other fungal species, such as *F. effusum*; but in this study, the intron was identified in all 125 isolates originating from pecan orchards in 16 counties across the state of Georgia. This provides a strong basis for the assumption that this intron is ubiquitous in the greater *F. effusum* population.

Characterization of this partial fragment of the *F. effusum* cyt *b* gene is of value as we assess the risk of QoI resistance occurring in isolates of this pathogen. The results of this study revealed that a group I intron of 1,407 bp occurs directly downstream of position 143, the presence of which prevents the occurrence of the G143A amino acid substitution known to confer complete resistance. Without the potential for this substitution, the risk of QoI resistance developing in the *F. effusum* populations of Georgia would seem to be relatively low. However, even with a reduced risk, resistance may still develop as a result of intron loss events facilitated by reverse transcriptase-like activity that may lead to the acquisition of the G143A substitution (Vallières et al. 2011). Additionally, selection pressure from repeated or long-term exposure to QoIs could lead to the loss of the intron and the evolution of intron-lacking genotypes harboring the G143A substitution like those observed in *B. cinerea* (Banno et al. 2009; Vallières et al. 2011).

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Table 2.1. The origin and number of *Fusicladium effusum* isolates collected from pecan in 2014 and used in this study.

County[a]	# of isolates	# of isolates with G137S[b]
Appling	1	0
Bleckley	5	0
Brooks	1	0
Burke	1	0
Crisp	8	1
Dougherty	8	1
Jeff Davis	1	0
Lanier	12	0
Lee	1	0
Lowndes	5	0
Mitchell	1	0
Peach	5	1
Stewart	1	1
Sumter	3	1
Terrell	3	1
Tift	69	1
TOTAL	125	7

[a] Counties in Georgia, USA.

[b] Isolates carrying the G137S amino acid substitution determined by comparing nucleotide sequences.

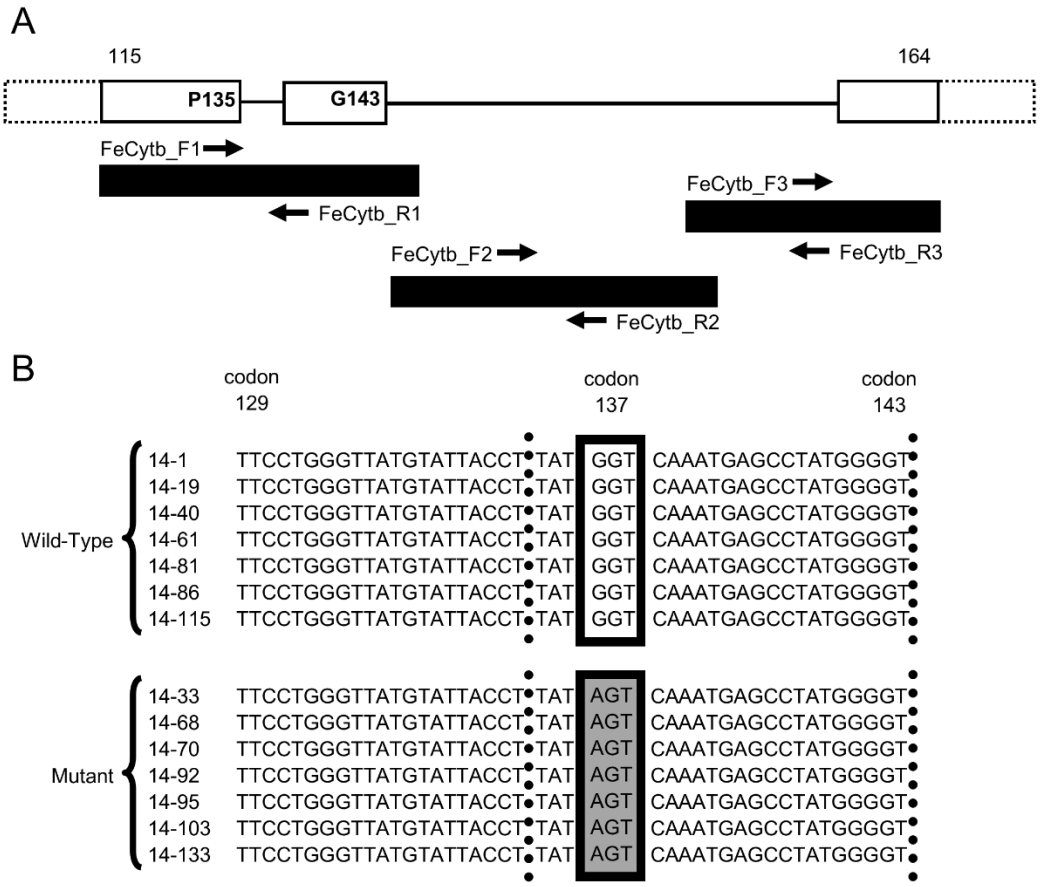

Figure 2.1. Partial characterization of the *Fusicladium effusum* cytochrome *b* (cyt *b*). **A.** Structure of the partial cyt *b* fragment. Empty boxes indicate exons and lines indicate introns. Dashed boxes represent non-sequenced parts of the gene. The solid black boxes illustrate the three overlapping amplicons used to identify the gene structure. Figure is not to scale. **B.** Partial nucleotide sequences of the cyt *b* gene. Codon 137 is boxed, the gray shading indicates the mutation responsible for the G137S amino acid substitution. The vertical dotted lines represent the position of the two introns indicated in Figure 2.1A.

CHAPTER 3

QUANTIFYING THE EFFECTS OF A G137S SUBSTITUTION IN THE CYTOCHROME *BC$_1$* OF *VENTURIA EFFUSA* ON AZOXYSTROBIN SENSITIVITY USING A DETACHED LEAF ASSAY[1]

[1]Standish, J. R., Brenneman, T. B., and Stevenson, K. L. 2019. Accepted by *Plant Disease*. Reprinted here with permission of the publisher, The American Phytopathological Society.

Abstract

The quinone outside inhibitor fungicides are known for their inherently high resistance risk due to substitutions in amino acid residues 129, 137, or 143 of the cytochrome *b* gene of phytopathogens. In *Venturia effusa*, cause of pecan scab, an intron adjacent to position 143 likely reduces this risk; however, the effects of a recently discovered substitution at position 137 (G137S) are unknown. Traditional in vitro assays are not useful for determining sensitivity of isolates of *V. effusa* to the QoI fungicides, due to the fungitoxic effects of required alternative oxidase inhibitors. A detached leaf assay was developed to quantify the sensitivity of 59 isolates to azoxystrobin: 45 wild-type and 14 carrying G137S. Isolate EC_{50} values ranged from <0.0001 to 0.3047 µg/ml; EC_{50} values for wild-type isolates ranged from <0.0001 to 0.2007 µg/ml (median 0.0023 µg/ml) while EC_{50} values for G137S isolates ranged from 0.0033 to 0.3047 µg/ml (median 0.0178 µg/ml). The median EC_{50} value for G137S isolates was significantly greater than that of the wild-type isolates; however, there was overlap between the two groups. This is the first report of sensitivity of *V. effusa* isolates to a QoI fungicide and evidence of G137S as a potential mechanism of partial resistance. However, although a complete control failure is unlikely, the impact of this substitution on QoI efficacy in Georgia pecan orchards remains to be determined.

Introduction

Management of pecan scab, caused by *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), relies on the application of preventive fungicides (Brock and Bertrand 2007a). This practice typically consists of 10 or more applications made every 10 to 14 days beginning at bud-break continuing until pollination and then every 14 to 21 days until shell hardening occurs (Brock and Bertrand 2007b). During periods of frequent rainfall, fungicides are often applied as frequently as every 7 to 10 days, thereby increasing the total number of applications during the growing season (Latham 1995; Brock et al. 2007). The quinone outside inhibitor (QoI; Fungicide Resistance Action Committee [FRAC] Code 11) fungicides have played a pivotal role in pecan scab management programs since the late 1990s as both stand-alone products and in formulated mixtures often in combination with a demethylation inhibitor fungicide (DMI; FRAC Code 3). There are a number of commercially available stand-alone QoI fungicides labeled for use on pecan in the United States that contain azoxystrobin (e.g., Abound 2.08F; Syngenta Crop Protection, Greensboro, NC), kresoxim-methyl (e.g., Sovran; BASF Corporation, Research Triangle Park, NC), or pyraclostrobin (e.g., Headline; BASF Corporation, Research Triangle Park, NC) (Brock et al. 2007).

The QoIs are mitochondrial respiration inhibitors that target the cytochrome bc_1 enzyme complex (Fernández-Ortuño et al. 2008). By binding to the quinol oxidation site of the enzyme complex, the fungicide effectively blocks electron transport, which halts ATP production and results in an energy deficit in fungal cells (Bartlett et al. 2002; Fernández-Ortuño et al. 2008; Gisi et al. 2002). The QoIs are considered to have an inherently high risk of resistance due to their site-specific mode of action and history of

resistance development in many plant pathogens (FRAC 2017). Since 1998, when QoI resistance was first identified in isolates of *Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* (Bartlett et al. 2002), practical resistance has been reported in more than 50 species representing 35 genera of both fungi and oomycetes (FRAC 2018). The mechanism of resistance to the QoI fungicides has typically been observed as a nucleotide point mutation in the cytochrome *b* (cyt *b*) gene that leads to an amino acid substitution at one of three positions in the encoded protein (Fernández-Ortuño et al. 2008). A change from glycine to alanine at position 143 (G143A) results in complete resistance and has been associated with complete control failures (Gisi et al. 2002). Partial resistance has been observed when substitutions of phenylalanine for leucine at position 129 (F129L) or glycine for arginine at position 137 (G137R) occur (Fernández-Ortuño et al. 2008; Gisi et al. 2002; Sierotzki et al. 2007). Cross resistance is frequently observed among members of this class of chemicals, such that isolates resistant to one QoI active ingredient are also resistant to all QoI active ingredients (Fernández-Ortuño et al. 2008; Sierotzki et al. 2000). An additional mechanism of resistance to the QoI fungicides may occur through the activation of an alternative oxidase respiration pathway (Fernández-Ortuño et al. 2008; Wood and Hollomon 2003). When this pathway is activated, mitochondrial electron transfer changes course and bypasses the QoI binding site, providing considerably less energy (~40%) to the pathogen (Wood and Hollomon 2003). As a result, this rescue mechanism has a low efficiency of energy conversion and does not support energy-demanding processes like conidial germination and host penetration that are important for infection (Fernández-Ortuño et al. 2008). This alternative pathway has been shown to limit curative QoI effectiveness in planta with established infections but

for the most part, AOX prevents QoI inhibition of spore germination in vitro (Fernández-Ortuño et al. 2008; Miguez et al. 2004; Wood and Hollomon 2003). Salicylhydroxamic acid (SHAM) and propyl gallate (PG) are typical inhibitors of AOX that are often used during in vitro studies of QoI sensitivity (Wood and Hollomon 2003).

A recent partial characterization of the *V. effusa* cyt *b* gene revealed the presence of a group I intron directly downstream of the glycine residue at position 143 (Standish et al. 2016). This finding indicates a reduced risk of QoI fungicide resistance as a mutation in codon 143 would prevent the correct excision of the intron and lead to a defective protein (Cech 1988; Lambowitz and Belfort 1993; Vallières et al. 2011). Practical evidence of this reduced risk of resistance has been observed in field studies where disease control from azoxystrobin was not compromised despite 20 consecutive applications over two years (Standish et al. 2018). These fungicides have been used on pecan since the late 1990s, but practical resistance has never been reported. However, efforts to accurately monitor QoI sensitivity have been hindered by the lack of a reliable screening method, as both SHAM and PG are fungitoxic to *V. effusa* in vitro (Seyran et al. 2010a, 2010b). Additionally, a single nucleotide polymorphism (G➔A) was recently identified in the first position of codon 137 leading to a novel amino acid substitution from glycine to serine (GGT➔AGT; G137S) (Standish et al. 2016). Thus, the objectives of this study were: to i) quantify QoI sensitivity in *V. effusa* using a detached leaf assay, and ii) determine the effects of the G137S amino acid substitution on QoI sensitivity in isolates of *V. effusa*.

Materials and Methods

Isolate collection and preparation. Samples of pecan leaves with actively sporulating pecan scab lesions were collected from two unmanaged baseline orchards in 1997 (orchards with no known history of fungicide exposure), eight managed commercial orchards in 2014, and an experimental orchard, in 2014, 2016, and 2017, with a long history of fungicide use. Monoconidial isolates of *V. effusa* (n=59) (Table 3.1) were established from individual leaf lesions following two different protocols, one described by Reynolds et al. (1997) and one outlined by Seyran et al. (2010a).

For samples collected from the two baseline orchards, a single pecan scab lesion was removed from each sampled leaf with a cork borer and the sporulating side of the lesion was rubbed gently across the surface of 4% water agar (WA) amended with 50 mg/liter each of chloramphenicol, streptomycin sulfate, and tetracycline in petri plates (Reynolds et al. 1997). The plates were incubated in the dark for 18 to 24 h before individual germinated conidia were transferred to petri plates containing potato dextrose agar made with the previously stated antibiotic amendments (PDA+) and then incubated at 25ºC in the dark to encourage mycelial growth (Reynolds et al. 1997). Colonized agar plugs were transferred to sterile glass tubes and covered with sterile deionized water amended with the previously described antibiotics and 0.1% Tween 20 (SDW+). The glass tubes were stored at room temperature for approximately 20 years before plugs were removed, placed onto PDA+, and incubated in the dark at 25ºC to allow for colony growth.

Samples collected from commercial orchards were submitted by pecan growers as part of an orchard-specific fungicide sensitivity monitoring program (Stevenson et al.

2015); while experimental orchard samples were collected as part of the aforementioned monitoring program (2014) or from individual trees throughout the course of a separate study (2016 and 2017) (Standish et al. 2018). In both cases, three groups of 15 leaflets were arbitrarily selected from each sample and conidia were collected by gently pumping SDW+ onto the surface of a sporulating lesion, on each leaflet, with a micropipette (Seyran et al. 2010a). These bulk conidial suspensions from groups of 15 leaflets were spread across the surface of 2% WA amended with the previously mentioned antibiotics, using a sterile glass rod and incubated for 24 h at room temperature (23 to 25ºC). Similar to the method described by Reynolds et al. (1997), germinated conidia were then transferred to petri plates containing PDA+ and incubated at 25ºC in the dark for 6 to 8 weeks to allow for colonies to develop (Seyran et al. 2010a).

Molecular characterization of *V. effusa* isolates. Genomic DNA was extracted and purified from mycelia of the *V. effusa* isolates (n=59) obtained from samples collected in 1997, 2014, 2016, and 2017 using an UltraClean Microbial DNA Isolation Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. A polymerase chain reaction (PCR) protocol and primers FeCytb_F1 and FeCytb_R1, previously described by Standish et al. (2016) was used to amplify a fragment of the *V. effusa* cytochrome *b* gene, detect mutations at positions 129, 137, and 143, and to confirm the presence of two introns within the gene fragment (Standish et al. 2016). PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sent to Eurofins MWG Operon LLC, Louisville, KY for custom sequencing. Genious version 11.0.5 and ClustalW within MEGA6 were used for analysis of nucleotide sequences (Kearse et al. 2012; Tamura et al. 2013).

Azoxystrobin sensitivity. A detached leaf assay was used to determine the sensitivity of each isolate to azoxystrobin. Two- to three-year-old pecan trees of cultivar Wichita grafted onto seedling rootstocks were obtained from a commercial nursery. Trees were pruned as necessary and planted in plastic tree pots (25 cm diameter) containing Miracle-Gro potting soil (Scotts Company, Marysville, OH). Except for a dormancy period (November 2017 through March 2018), during which trees were kept outside under ambient conditions, the trees were maintained in a greenhouse, watered biweekly, and fertilized twice per year with a 15N-9P-12K slow release fertilizer (Osmocote Plus; Scotts Company, Marysville, OH). Susceptible leaflets were removed from trees and stored at 4ºC for up to 1 week before use. Trees were pruned and defoliated periodically to stimulate the production of susceptible leaf tissue for use in the study.

Azoxystrobin (as Abound 2.08F; Syngenta Crop Protection, Greensboro, NC) was suspended in sterile deionized water and serial dilutions were prepared to obtain final azoxystrobin concentrations of 0.0, 0.0001, 0.001, 0.01, 0.1, or 1 µg a.i./ml. The suspensions were applied to the upper surface of the leaflets until run-off using an aerosol propellant atomizer (Crown Spra-Tool; Aervoe Industries, Inc., Gardnerville, NV). The leaflets were allowed to air dry before a single leaflet was placed onto the surface of 2% WA amended with 40 µg/ml benzimidazole for each replicate of each concentration. Leaflets were secured by inserting the leaf base and tip into the agar medium (Cancro 2000).

Conidia for bioassays were produced by placing two hyphal plugs (5-mm diameter) into sterile microcentrifuge tubes containing 1 ml potato dextrose broth

amended with the previously mentioned antibiotics (PDB+) and three sterile 3-mm glass beads. Fungal material was macerated using a bead beating apparatus (Mini-Bead Beater, Biospec, Bartlesville, OK) for 1 min and transferred into tubes containing 6 ml PDB+. This fungal slurry was mixed with a vortex mixer and transferred onto multiple petri plates (15 × 60 mm diameter) containing oatmeal agar amended with the previously mentioned antibiotics, in aliquants of 1 ml, and spread across the surface of the media using a sterile glass rod. Plates were placed in an incubator set at 25ºC with a 14-h day/night cycle provided by fluorescent light sources. After 10 to 14 days, conidia were harvested by flooding the plates with 3 ml of SDW+. The resulting conidial suspension was centrifuged at 6500 RPM for 6 min, the supernatant decanted, and the conidial pellet resuspended in 1.5 ml of SDW+ (Cancro 2000). This solution was filtered through two layers of sterile cheese cloth and adjusted to a concentration of 1×10^5 conidia/ml using a hemocytometer (Turechek and Stevenson 1998). A micropipette was used to place 50 µl of the previously prepared *V. effusa* conidial suspension onto each leaflet. The inoculated leaflets were incubated at 25ºC in the dark for 48 h (Cancro 2000). Conidial germination was determined for 50 conidia per leaflet using a compound microscope (×200). A conidium was recorded as germinated if the length of the germ tube was at least four times the diameter of the conidium or if an appressorium was visible. For each isolate, the sensitivity tests were conducted twice and in groups of ten isolates at a time. However, throughout the study, six different isolates of *V. effusa* were included as internal controls and were represented in at least five separate experiments each.

Data analysis. The concentration of azoxystrobin that effectively inhibited conidial germination by 50% (EC_{50}) was estimated for each isolate (n=59) by linearly

regressing relative inhibition on log_{10}-transformed fungicide concentrations using PROC REG in SAS (v. 9.4, SAS Institute, Cary, NC). Residuals and quantile plots were examined, and a Shapiro-Wilk test was performed in PROC UNIVARIATE to assess normality of log_{10}-EC_{50} values for both wild-type isolates (n=45) and those carrying the G137S amino acid substitution (n=14). The log_{10}-EC_{50} values of wild-type isolates were compared by year of collection (1997, 2014, 2016, and 2017) using a non-parametric, Kruskall-Wallis test performed with PROC NPAR1WAY in SAS. Likewise, the distributions of log_{10}-EC_{50} values of all wild-type isolates were compared to that of the G137S isolates using a non-parametric, two-sided Wilcoxon Rank Sum Test, also performed in SAS. The coefficient of variability of log_{10}-EC_{50} values (standard error of log_{10}-EC_{50} values divided by mean of log_{10}-EC_{50} values) for the six internal control isolates was calculated and used to assess reproducibility of the assay.

Results

Molecular characterization of *V. effusa* isolates. The primers amplified a DNA fragment of approximately 600 bp and sequence analyses confirmed the presence of a portion of the 1,407-bp intron as well as the presence of the previously described 360-bp intron downstream of position 135 in all 59 isolates. Phenylalanine (F) and glycine (G) were present at positions 129 and 143, respectively, in all 59 isolates. The previously described G137S substitution was confirmed in the cyt *b* gene of 14 isolates as a mutation of guanine replaced by adenine in the codon at positon 137 (GGT➔AGT) (Standish et al. 2016). Partial nucleotide sequences of the cyt *b* gene for the 14 *V. effusa* isolates carrying the G137S substitution (ST11A, ST12A, TE9A, TE9B, TE9C, TE9D, TE9E,

TE11A, TE11B, TE11C, TE11D, TE11E, CR5A, and CR5B) and four wild-type *V. effusa* isolates (JD-97-20, JD-97-22, TC8, and TC38) were deposited into GenBank (Acc. No. MH647746 – MH647763).

Azoxystrobin sensitivity. For each of the six internal control isolates of *V. effusa* examined in at least five experimental repeats of this assay, the mean EC_{50} values ranged from 0.0004 to 0.0138 µg/ml and coefficients of variability ranged from 0.0350 to 0.1080 (Table 3.2). The mean EC_{50} value for these six isolates was 0.0053 µg/ml with a mean coefficient of variability of 0.0662 (Table 3.2). All coefficients of variability were less than 0.2 indicating that the results for the isolates were consistent among experimental repeats. Therefore, the mean EC_{50} values for the remaining isolates screened in this study were determined based on combined data from all experimental repeats. Frequency distributions of the mean EC_{50} values were assessed for normality, but for 10 of the 45 wild-type isolates, EC_{50} values could not be estimated (values <0.0001 µg/ml). When those 10 isolates were excluded from the analysis, the assumptions of normality for log_{10}-EC_{50} values of the remaining isolates were met ([Pr < W] = 0.7712). The distribution of mean log_{10}-EC_{50} values for isolates carrying G137S was also normal ([Pr < W] = 0.1327). The mean EC_{50} values for the 59 isolates screened for their sensitivity to azoxystrobin ranged from a minimum of <0.0001 to a maximum of 0.3047 µg/ml (Table 3.3). Year of collection was not a significant factor for wild-type isolates (P = 0.1354), thus the baseline isolates included in this study (n=15) were combined with the remaining wild-type isolates (n=30) for further analysis. Mean EC_{50} values for wild-type isolates ranged from <0.0001 to 0.2007 µg/ml while those with G137S ranged from 0.0033 to 0.3047 µg/ml (Fig. 3.1; Table 3.3). Overall, isolates with G137S were approximately

eight times less sensitive to azoxystrobin than the wild-type isolates, based on the respective medians, which differed significantly according to the two-sided Wilcoxon Rank Sum Test ($P = 0.0010$) (Table 3).

Discussion

All fungicides labelled for pecan scab management have some inherent risk of resistance, ranging from "low to medium" to "high" (FRAC 2017). Practical resistance or significant reductions in sensitivity has been documented for the DMIs, methyl benzimidazole carbamates, and the organotins (Littrell 1976, Reynolds et al. 1997; Seyran et al. 2010a; Standish et al. 2018; Stevenson et al. 2004, 2015). After nearly two decades of use, practical resistance to the QoIs has never been reported despite their high risk for resistance to develop. Similarly, shifts in QoI sensitivity have never been reported, however, the fungitoxic effects of SHAM and PG on *V. effusa* in vitro have thwarted monitoring efforts. The recent discovery of a single nucleotide polymorphism in the first position of codon 137 of the *V. effusa* cyt *b* paired with the inability of traditional in vitro bioassays (i.e. spore germination/mycelial growth assays) to accurately quantify QoI sensitivity underscore the importance of our efforts to develop this alternative screening method. In this study, we developed a detached leaf assay for use in quantifying sensitivity of *V. effusa* isolates to azoxystrobin without the need for an AOX inhibitor because the alternative respiration pathway is not known to interfere with QoI-activity against conidial germination in planta. This reproducible method has a known level of precision and was used to generate the first frequency distribution of *V. effusa* sensitivities to a member of this important chemical class. Although labor intensive and

time-consuming, the assay and the results generated herein may be used in the future to detect potential shifts in sensitivity to azoxystrobin within *V. effusa* populations. Additionally, this is the first characterization of sensitivity to a QoI fungicide for isolates of *V. effusa* or any phytopathogen carrying the G137S amino acid substitution reported previously (Standish et al. 2016).

The distribution of mean EC_{50} values calculated for all 59 isolates assayed in this study was unimodal and slightly skewed to the right with 10 isolates that exhibited EC_{50} values less than the lowest concentration tested (0.0001 µg/ml) (Fig. 3.1). Isolate sensitivity did differ significantly when comparing wild-type and G137S isolates. Based on the respective median EC_{50} values, G137S isolates were approximately eight times less sensitive than that of the wild-type isolates; however, there was some overlap between the respective sensitivity distributions (Fig. 3.1). This reduction in sensitivity of G137S isolates of *V. effusa* is consistent with the reduction in sensitivity observed in isolates of *Pyrenophora tritici-repentis* (Died.) Drechsler exhibiting both F129L and G137R (Siertozki et al. 2007). Unlike the complete resistance associated with the G143A substitution, the partial resistance reported in isolates with the F129L substitution does not appear to have the same level of impact on fungicide efficacy (Pasche et al. 2005; Sierotzki et al. 2007). A study examining the effects of azoxystrobin on disease caused by F129L isolates of *Alternaria solani* Sorauer (n=5) revealed that significant reductions in efficacy may occur, though, this was not consistent for all isolates tested (Leiminger et al. 2013). Additionally, field studies in Minnesota and North Dakota examining control of early blight of potato have shown that QoI fungicides have not performed better than older, multi-site fungicides (i.e., chlorothalonil or mancozeb) when the pathogen

population is dominated by F129L isolates (95.7% of 1212 isolates in Minnesota and 99.3% of 1300 isolates in North Dakota, from 2002-2006) (Pasche and Gudmestad 2008). To date, studies testing QoI efficacy on trees inoculated with G137S isolates of *V. effusa* have not been conducted and merit further examination.

In both the case of *P. tritici-repentis* (Sierotzki et al. 2007) and the isolates of *V. effusa* used in this study, the amino acid substitution at residue 137 was caused by a change in the first nucleotide of the codon (GGT➔AGT). In non-phytopathogenic organisms, single nucleotide changes leading to G137V or G137E substitutions have been linked with a reduced respiratory capacity (Brasseur et al. 1996; Fisher and Meunier 2001). Thus, a mutation at this position may result in a potential fitness penalty in the pathogen that could prevent its long-range distribution or ability to dominate the overall population (Brasseur et al. 1996; Fisher and Meunier 2001; Gisi et al. 2002). The G137S isolates identified in this study were obtained from samples collected from four managed pecan orchards in three Georgia counties; these samples had 100% frequency of G137S isolates (Crisp County – 2/2; Stewart County – 2/2; Terrell County – 5/5 and 5/5, respectively) (Table 3.1). These isolates represent approximately 24% of all isolates assayed in this work, although selection for use herein was based on presence or absence of the mutation and is not likely representative of the overall population. In our previous work, based on extensive state-wide sampling, seven of 125 isolates carried G137S (approximately 6%) which is likely to be a more accurate representation of its prevalence across Georgia pecan orchards (Standish et al. 2016). However, the prevalence of G137S in individual pecan orchards is not known but may lead to an impact on efficacy as in *A. solani* populations dominated by the F129L substitution (Pasche and Gudmestad 2008).

Previous research characterizing a portion of the *V. effusa* cyt *b* gene revealed a group I intron directly downstream of position 143 in 125 isolates (Standish et al. 2016). Due to the mechanism by which group I introns are removed from mRNA transcripts, a nucleotide mutation like that which causes the G143A substitution would be lethal as the result of a non-functional protein and such mutants would disappear from the population (Cech 1988; Lambowitz and Belfort 1993; Vallières et al. 2011). A 2-year field study of fungicide efficacy for pecan scab control provided practical evidence of this expected reduced risk of resistance (Standish et al. 2018). After ten consecutive applications per season, Abound 2.08F (azoxystrobin) and Abound 2.08F plus Orius 3.6F (azoxystrobin plus tebuconazole) resulted in less disease than most other fungicide treatments included in the study (Standish et al. 2018). Several of the isolates assayed in the present study were collected from the experimental orchard where these products were tested in 2016 and 2017 (Tift County; Table 3.1). The mean EC_{50} values for these isolates were similar to those of the baseline isolates collected in 1997 (Table 3.1), despite the consistent selection pressure exerted on the population. Additionally, none of those isolates carried the G137S substitution. As mentioned previously, there have been no reports of practical resistance to QoI fungicides in the pecan scab pathogen even though several fungicides in this group have been available for pecan since the late 1990s; however, monitoring sensitivity had been difficult until now.

In developing this detached leaf assay, we now have a reproducible method for quantifying QoI sensitivity in *V. effusa*. The results generated here are useful for future monitoring efforts to identify shifts in sensitivity when or if they occur. Isolates with the G137S amino acid substitution were less sensitive than the wild-type isolates but the

impact of these mutants on QoI efficacy in the field is unknown. Based on the sequence results, all 59 isolates tested in this study possessed the group I intron downstream of position 143. This indicates that an amino acid substitution at position 143 would be lethal and provides further evidence of a reduced risk of QoI resistance in *V. effusa* (Standish et al. 2016). However, resistance may still develop after repeated or long-term exposure if intron-lacking genotypes occur and are selected for, or additional and as-of-yet-unknown mechanisms of resistance are identified (Fernández-Ortuño et al. 2008; Vallières et al. 2011).

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Table 3.1. Georgia county of origin and number of isolates of *Venturia effusa* collected from pecan and used in this study

			No. of isolates	
Year	**County**	**Orchards[a]**	**G137S[b]**	**Collected**
1997	Jeff Davis	1	0	9
	Troup	1	0	6
2014	Crisp	2	2	3
	Dougherty	1	0	1
	Lanier	2	0	5
	Peach	1	0	4
	Stewart	2	2	2
	Terrell	2	10	10
	Tift	1	0	6
2016	Tift	1	0	8
2017	Tift	1	0	5
Total			14	59

[a]Number of sampled orchards within each county of origin

[b]*Venturia effusa* isolates with the AGT at codon 137 previously described by Standish et al. (2016).

Table 3.2. Reproducibility of effective concentration of azoxystrobin at which conidial germination was inhibited by 50% (EC_{50} values) determined for six internal control isolates of *Venturia effusa* using a detached leaf assay

	Azoxystrobin EC_{50} (µg/ml)			
Isolate	**Mean**	**Median**	**Range**	**CV[a]**
JD-97-20	0.0024	0.0029	0.0009-0.0032	0.0366
JD-97-9	0.0004	0.0003	0.0001-0.0007	0.0350
P16AT3	0.0138	0.0084	0.0014-0.0491	0.1080
P16AT4	0.0025	0.0018	0.0004-0.0059	0.0685
TC8	0.0012	0.0004	0.0001-0.0042	0.0851
TE9B	0.0117	0.0071	0.0043-0.0279	0.0642
Mean	0.0053	0.0035	0.0012-0.0152	0.0662

[a]CV = Coefficient of variability expressed as the absolute value of (standard error of $\log_{10}$-EC_{50} values)/(mean of $\log_{10}$-EC_{50} values).

Table 3.3. Median and range of EC_{50} values for isolates of *Venturia effusa* to azoxystrobin

			EC_{50} (µg/ml)		
Isolates	**Codon 137[y]**	**No. of isolates**	**Median**		**Range**
Wild Type	GGT	45	0.0023	b[z]	<0.0001-0.2007
G137S	AGT	14	0.0178	a	0.0033-0.3047
Combined	-	59	0.0043		<0.0001-0.3047

[y]Nucleotide sequence of codon 137 of the *Venturia effusa* cytochrome *b* gene.
[z]Different letters indicate significant differences between medians based on the Wilcoxon Rank Sum Test (P=0.0010).

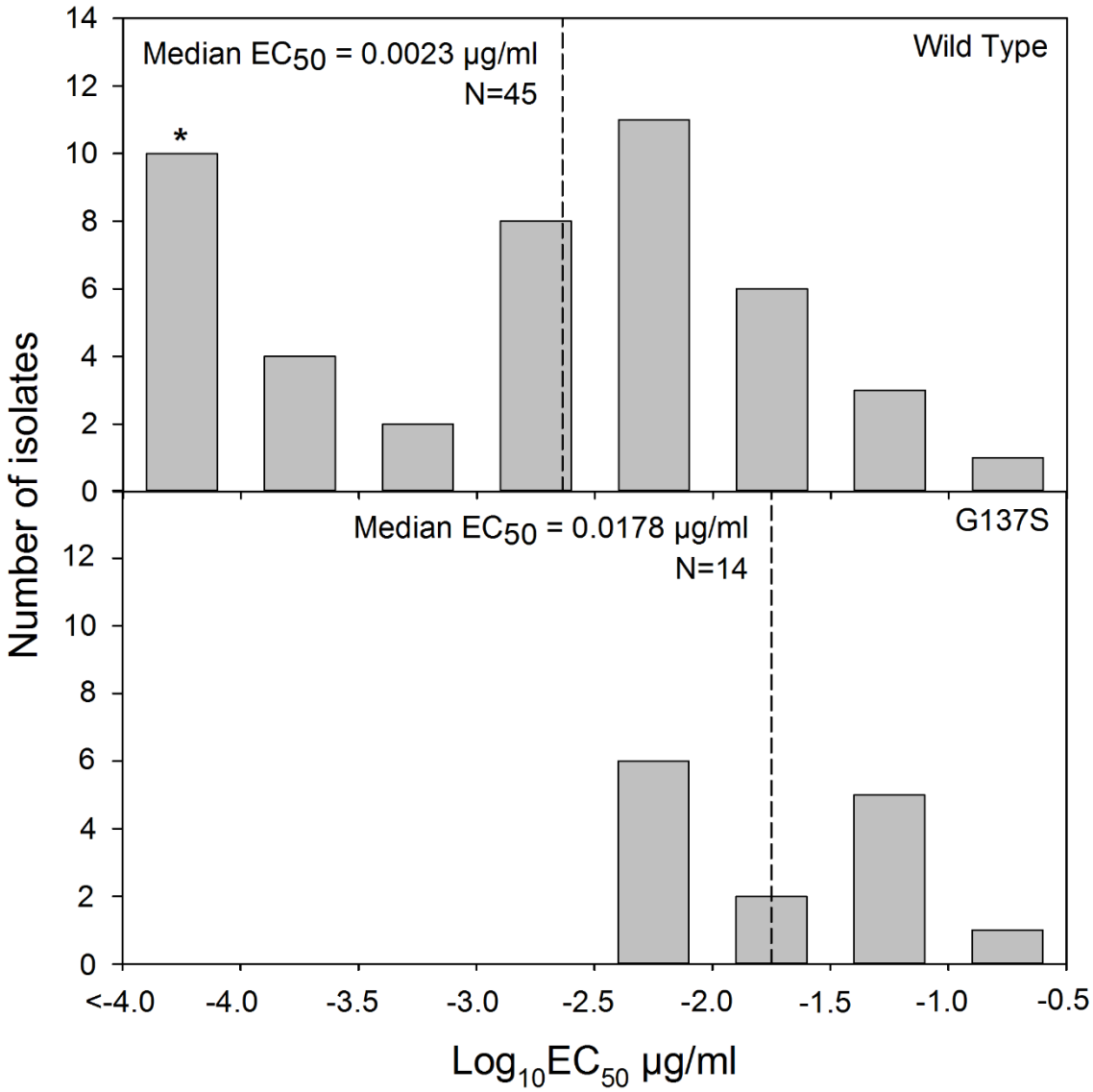

Figure 3.1. Frequency distribution of effective concentration of azoxystrobin at which conidial germination was inhibited by 50% (EC_{50} values) for isolates of *Venturia effusa* carrying sequences coding for either glycine (Wild type; top) or serine (G137S; bottom) at position 137 of the cytochrome *b* gene. Bars are positioned on the mid-point of each respective histogram bin. Vertical dashed lines represent median $log_{10}EC_{50}$ values. The asterisk (*) indicates 10 isolates with EC_{50} values less than the lowest concentration tested in this study.

CHAPTER 4

DYNAMICS OF FUNGICIDE SENSITIVITY IN *VENTURIA EFFUSA* AND FUNGICIDE EFFICACY UNDER FIELD CONDITIONS[1]

[1]Standish, J. R., Brenneman, T. B., and Stevenson, K. L. 2018. Accepted by *Plant Disease*. Reprinted here with permission of the publisher, The American Phytopathological Society.

Abstract

Venturia effusa, which causes scab, has developed resistance to fungicides that were once effective. Over 2 years, laboratory-based sensitivity of fentin hydroxide (TPTH) and tebuconazole in *V. effusa* and their efficacy under field conditions were compared. Leaf and nut scab were assessed on trees receiving ten applications of TPTH, tebuconazole, azoxystrobin, azoxystrobin plus tebuconazole, TPTH plus tebuconazole, or no fungicide (NTC) per year. Sensitivity of *V. effusa* on leaflets collected from treated and nontreated trees was assessed in June and September, respectively. The mean relative germination (RGe) on 30 µg/ml TPTH was 10.9% and 40.9% in 2016, and 4.2% and 0.6% in 2017. Mean relative growth (RGr) on 1 µg/ml tebuconazole in 2016 was 45.5% and 34.6%, and 69.3% and 56.3% in 2017. In both years, leaf and nut scab were significantly lower on trees treated with azoxystrobin, azoxystrobin + tebuconazole, or TPTH + tebuconazole when compared to NTC and tebuconazole-treated trees. Compared with the NTC, tebuconazole did not significantly reduce leaf scab in 2017 or nut scab in either year, indicating that an RGr value between 34.6% and 69.3% is likely to result in a control failure on tebuconazole-treated trees. Although better activity was expected, TPTH reduced scab with RGe values between 0.6% and 40.9%. These results are valuable for developing fungicide sensitivity thresholds to better predict fungicide performance.

Introduction

Pecan scab, caused by *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), is the most economically important disease affecting pecan [*Carya illinoinensis* (Wangenh.) K. Koch] in the southeastern United States (Demaree 1924). This fungus causes small olive-brown to black spots on infected leaves, fruit shucks, and twigs; young and actively growing tissues are most susceptible to infection and become resistant as they mature (Demaree 1924; Littrell and Bertrand 1981). Infections occurring on fruit during the early stages of development can cause premature fruit drop, but those occurring after shell hardening are thought to be cosmetic (Bertrand 2002; Demaree 1924; Hunter 1983). The annual severity of scab is determined by cultivar susceptibility, the relative abundance of *V. effusa* inoculum, and the frequency of spring and summer rainfall (Latham 1982, Sparks et al. 2009). Rainfall events aid in the dispersal of conidia by wind and splash and the ensuing foliar moisture provides optimal conditions for conidial germination and infection (Gottwald 1982; Gottwald 1985; Gottwald and Bertrand 1982; Latham 1982; Turechek and Stevenson 1998).

The most effective method of managing scab is through the use of resistant cultivars (Conner and Wells 2007). However, the scab pathogen exhibits a great amount of genetic and pathogenic diversity which has allowed for its adaptation to cultivars with scab resistance (Bock et al. 2014, 2017a; Conner and Stevenson 2004; Demaree and Cole 1929; Sparks 1992). Thus, in the absence of durable host resistance, scab management is centered on preventive fungicide applications made throughout the growing season (Brock and Bertrand 2007a). In Georgia, the general guideline is to apply fungicides on 10- to 14-day intervals from bud break to pollination, and on 14- to 21-day intervals from

pollination to shell hardening, resulting in 10 or more applications per season (Brock and Bertrand 2007b). However, the interval between sprays is often adjusted to once every 7 to 10 days during periods of frequent rainfall, which substantially increases the total number of applications per growing season (Latham 1995; Brock et al. 2007). Fungicides from different chemical groups are approved for use on pecan in the U.S. and include the methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), organotin compounds, phosphonates, dithiocarbamates, and guanidines (Fungicide Resistance Action Committee [FRAC] Code 1, 3, 11, 30, 33, M3, and U12, respectively) (Bock et al. 2017b). The inherent risk of resistance developing in *V. effusa* to the active ingredients within each of these fungicide groups ranges from "low to medium" to "high" and is magnified by the difficulty of providing uniform coverage in larger pecan trees (Bertrand and Brenneman 2001; Bock et al. 2013, 2015; FRAC 2017).

Practical fungicide resistance in *V. effusa* was first observed in the mid-1970s in association with benomyl control failures in Alabama and Georgia (Littrell 1976). Since that time, significant reductions in sensitivity have been reported in *V. effusa* isolates to the organotin fentin hydroxide in vitro and to the DMIs, both in vitro and as observed control failures in some locations (Reynolds et al. 1997; Seyran et al. 2010; Stevenson et al. 2004, 2015). Subsequently, a fungicide monitoring program was initiated in 2008, and further expanded in 2014 and 2015, allowing Georgia pecan producers to submit leaf samples showing typical scab symptoms for fungicide sensitivity testing (Seyran 2010; Stevenson et al. 2015). Sensitivity was determined by collecting conidia directly from infected leaf material and testing either germination or micro-colony growth on medium

amended with dodine, fentin hydroxide, thiophanate-methyl, propiconazole, or tebuconazole, using a rapid in vitro method (Seyran et al. 2010). This assay utilized discriminatory concentrations of the aforementioned active ingredients to identify insensitivity when compared to a baseline population (Seyran et al. 2010). Studies utilizing similar in vitro laboratory assays provide an indication of relative sensitivity or insensitivity of an isolate or sample to a particular fungicide active ingredient and can be useful for detecting shifts in sensitivity over time. However, a decrease in sensitivity to a fungicide in vitro over time does not necessarily mean that practical resistance has developed or will develop if in vitro threshold levels have not yet been determined for individual fungicides. Understanding the relationship between fungicide efficacy in the orchard and sensitivity levels as measured in the laboratory is essential for development of fungicide recommendations and fungicide programs for effective scab management. The objectives of this research were to examine the within season changes in sensitivity to fentin hydroxide (TPTH) and tebuconazole in *V. effusa* as influenced by fungicide treatment and to explore the relationship between fungicide sensitivity and efficacy in managing pecan scab under field conditions.

Materials and Methods

Field experiment and sample collection. Field experiments were conducted in a pecan orchard at the University of Georgia Ponder Farm located in Ty Ty, GA in 2016 and 2017. This orchard was originally established in 1988 with alternating rows of cv. Desirable and cv. Wichita planted in a square pattern with approximately 12.2 m between trees both within and across rows. Every other tree in all rows of both cultivars had been

replanted in 2009, and these younger trees were used in this study. Each tree in the study was bordered on all sides by an older tree that was also sprayed for scab with a program consisting primarily of Super Tin and Elast (dodine). Data were collected from cv. Wichita, a cultivar known for being highly susceptible to scab. Fungicide treatments were applied using an air-blast sprayer in a volume of 950 L/Ha every 14 days for a total of ten applications beginning at bud break (18 April 2016; 8 April 2017). While not a recommended use pattern, this strategy allowed for a direct evaluation of the efficacy of each treatment of interest. The experiment was arranged in a randomized complete block design with six treatments and five replications (blocks). The six treatments were Super Tin 4L [fentin hydroxide (TPTH); United Phosphorus Inc., King of Prussia, PA], applied at a rate of 0.877 L/ha; Orius 3.6F (tebuconazole; ADAMA Americas Inc., Aventura, FL), applied at a rate of 0.585 L/ha; Abound (azoxystrobin; Syngenta Crop Protection, Greensboro, NC), applied at a rate of 0.877 L/ha; Abound plus Orius 3.6F, applied as a tank mixture at rates of 0.877 and 0.585 L/ha, respectively; Super Tin 4L plus Orius 3.6F, applied as a tank mixture at rates of 0.877 and 0.585 L/ha, respectively; and a nontreated control. Treatments were applied to the same trees in both years of the study.

Disease assessments were made on a bi-weekly basis for 8 weeks after leaf scab symptoms were observed in each year and on a monthly basis for 2 to 3 months after nut scab symptoms were observed. Eight arbitrarily chosen terminals in the lower canopy of each tree were evaluated per tree for incidence of leaf scab and incidence and severity of nut scab. Leaf scab incidence was evaluated as the number of leaflets exhibiting symptoms of scab divided by total number of leaflets. Nut scab incidence and severity were measured as the number of nuts showing symptoms of scab divided by total number

of nuts and by estimating the percent diseased nut shuck area ranging from 0-100%, respectively.

Fungicide sensitivity. In each year, leaflets exhibiting scab symptoms were first sampled when disease incidence was high enough to obtain an adequate sample (typically post-pollination) and again just prior to shuck-split. Approximately 50 leaflets were collected from each of the 30 trees in the study on 22 June and 15 September in 2016 and 15 June and 8 September in 2017. Leaflets were placed in plastic bags and refrigerated (4ºC) until they were processed, typically within 5 days. To confirm sporulation and pathogen identity, leaf lesions were examined using a stereomicroscope to identify *V. effusa* conidia prior to testing.

Sensitivity to TPTH and tebuconazole was estimated using a slightly modified assay first described by Seyran et al. (2010). Technical-grade TPTH (98.7% a.i.; Chem Service, Inc., West Chester, PA) and tebuconazole (97.5% a.i.; Bayer CropScience, Research Triangle Park, NC) were each dissolved in acetone to reach concentrations of 30,000 µg/ml and 1,000 µg/ml, respectively. A final concentration of 30 µg/ml TPTH in media was obtained by adding 1 ml/liter of the TPTH solution to autoclaved 2% water agar (WA; 20 g Bacto agar per liter of water) that had cooled to 50°C. Similarly, the final concentration of 1 µg/ml tebuconazole in media was obtained by adding 1 ml/liter of the tebuconazole solution to autoclaved quarter-strength potato dextrose agar (qPDA; 9.75 g PDA and 11.25 g Bacto agar per liter of water), cooled to 50°C. A non-fungicide amended control containing 1 ml/liter of acetone was included for both fungicides (Seyran et al. 2010). The prepared medium was poured into disposable petri plates (15 × 100 mm diameter) and refrigerated in the dark at 4ºC for use within 3 days.

Each sample of leaflets per tree was divided into three groups and 15 individual lesions were selected from each group for a total of 45 lesions per sample. A 9-µl droplet of sterile deionized water solution amended with antibiotics and Tween 20 (50 µg/ml each of chloramphenicol, streptomycin sulfate, and tetracycline, and one drop Tween 20 per 100 ml, respectively) was placed on to each lesion and conidia were dislodged and collected. Conidia from each group of 15 lesions were combined in a 1.5-ml microcentrifuge tube and suspensions were mixed well with a vortex mixer (Seyran et al. 2010).

To determine sensitivity to TPTH, 23 µl of each of the three conidial suspensions per sample were placed onto two replicate plates of TPTH-amended and non-amended WA. Conidia were incubated in the dark at 25ºC ± 2 for 48 h, and conidial germination was assessed for 50 arbitrarily selected conidia per replicate. A conidium was considered germinated if the length of the germ tube was at least four times the length of the conidium itself. Percent relative germination (RGe) was calculated as the proportion of germinated conidia on the fungicide-amended medium divided by the proportion of germinated conidia on non-amended medium, multiplied by 100. This value was generated for each of the three groups, which were then averaged to yield a mean RGe value for each sample (Seyran et al. 2010).

To determine sensitivity to tebuconazole, 23 µl of each of the three conidial suspensions per sample were placed onto two replicate plates of tebuconazole-amended and non-amended qPDA. Conidia were spread using a sterile glass rod and incubated in the dark at 25ºC ± 2 for 72 h (Seyran et al. 2010). After incubation, plates were examined under a compound microscope (×100) and ten single, well-separated colonies

were arbitrarily selected and the maximum diameter of each colony was measured. The DMI fungicides are not expected to inhibit conidial germination but are expected to inhibit hyphal growth. To correct for the average combined length of a germinated spore and the germ tube, 15 µm were subtracted from all micro-colony diameter measurements. Percent relative growth (RGr) was calculated as the corrected mean diameter of ten micro-colonies on the fungicide-amended medium divided by the corrected mean diameter of ten micro-colonies on the non-amended medium, multiplied by 100. This RGr value was calculated for each of the three groups, which were then averaged to yield a mean RGr value for each sample (Seyran et al. 2010).

Data analysis. Statistical analyses were performed separately for 2016 and 2017 datasets. A mixed model analysis was conducted using PROC GLIMMIX in SAS (SAS version 9.4, Cary, NC) to examine the effects of fungicide programs on area under the disease progress curve, maximum leaf scab incidence, maximum nut scab incidence, and maximum nut scab severity. For each of these four response variables, fungicide treatment was treated as a fixed effect with block as a random effect. The residuals for area under the disease progress curve, maximum leaf scab incidence, maximum nut scab incidence, and maximum nut scab severity met the assumptions of normally distributed data. A repeated measures analysis of variance was performed in PROC GLIMMIX to identify whether sampling date, fungicide treatment, or sampling date by fungicide treatment significantly impacted fungicide sensitivity of *V. effusa* on TPTH or tebuconazole amended medium. Tebuconazole sensitivity (RGr) residuals met the assumptions of normally distributed data. However, the residuals of TPTH sensitivity

(RGe) were non-normal and as such, those data were analyzed using the lognormal distribution based on (RGe + 1) to avoid taking the logarithm of 0.

Results

Leaf scab symptoms were first observed in late May of both 2016 and 2017; however, scab pressure and epidemic development were vastly different between the two years (Fig. 4.1). In 2016, environmental conditions were conducive for early season scab infections with a cumulative rainfall between 1 March and 31 May of 362 mm over 18 events compared with 199 mm over 11 events in 2017 (Appendix A, Table A.1). This 2016 spring rainfall resulted in greater amounts of leaf scab across all treatments (Table 4.1). Conversely, cumulative rainfall between 1 June and 31 August in 2016 was 256 mm over 17 events compared with 379 mm over 28 events in 2017. The environment in 2017 was not conducive for early season infection which was reflected in the lower maximum leaf scab incidence and area under the disease progress curve values on the nontreated control trees (Table 4.1). The greater summer rainfall in 2017 led to first appearance of nut scab symptoms approximately 4 weeks earlier than in 2016 and maximum nut scab severity was greater on the nontreated control trees in 2017 than in 2016 (Fig. 4.1).

All fungicide treatments significantly reduced maximum leaf scab incidence and area under the disease progress curve when compared to the nontreated in 2016; however, Abound, Abound plus Orius 3.6F, and Super Tin 4L plus Orius 3.6F significantly reduced maximum leaf scab incidence and area under the disease progress curve when compared to the stand-alone Super Tin 4L and Orius 3.6F treatments (Table 4.1). In

2017, maximum leaf scab incidence and area under the disease progress curve were significantly lower in trees treated with Abound or Abound plus Orius 3.6F than in trees treated with all other fungicides. Trees that received the stand-alone Super Tin 4L or Super Tin 4L plus Orius 3.6F treatments had significantly reduced maximum leaf scab incidence and trees treated with these treatments or Orius 3.6F alone had significantly reduced area under the disease progress curve when compared to the nontreated trees. There were no significant effects of treatment on maximum nut scab incidence in either year but maximum nut scab severity was significantly reduced in both years on trees treated with Abound, Abound plus Orius 3.6F, Super Tin 4L, or Super Tin 4L plus Orius 3.6F when compared to the nontreated trees (Table 4.1).

For RGe values on TPTH-amended medium, the fungicide treatment by date of sampling interaction was not significant ($P = 0.3076$) nor was the effect of fungicide treatment alone ($P = 0.0626$); however, RGe values did differ significantly among sampling dates ($P < 0.0001$), therefore data were pooled over treatment and compared by date. For all fungicide treatments combined, insensitivity to TPTH increased significantly between 22 June and 15 September 2016, decreased significantly between 15 September 2016 and 15 June 2017, and again, decreased significantly between 15 June and 8 September 2017 (Table 4.2). Similarly, for RGr values on tebuconazole-amended medium, the interaction between fungicide treatment and sampling date and fungicide treatment alone were not significant ($P = 0.1079$ and $P = 0.8906$, respectively). As observed with TPTH RGe values, RGr values on tebuconazole differed significantly among sampling dates ($P < 0.0001$) and data were pooled over treatment and compared by date. Insensitivity to tebuconazole across all treatments decreased significantly

between 22 June and 15 September 2016, increased significantly between 15 September 2016 and 15 June 2017, and decreased significantly from 15 June to 8 September 2017 (Table 4.2).

Discussion

Studies utilizing in vitro bioassays may provide an indication of relative sensitivity or insensitivity of an isolate or sample to a particular active ingredient and are useful for detecting shifts in sensitivity over time. However, a decrease in sensitivity to a fungicide in vitro does not necessarily indicate the occurrence of practical resistance. The goals of this study were to examine the within season changes in sensitivity to TPTH and tebuconazole in *V. effusa* as influenced by fungicide treatment and to explore the relationship between fungicide sensitivity and efficacy in managing pecan scab under field conditions. As important members of the scab management arsenal, products containing these two active ingredients are heavily relied upon by pecan producers; therefore, monitoring and managing the development of resistance are very important to preserve their efficacy. The concentrations of TPTH and tebuconazole used in the sensitivity assays were initially selected based on a baseline study conducted by Seyran et al. (2010). For TPTH, the probability of an isolate sampled from the baseline population having an EC_{50} value greater than the discriminatory concentration of 30 µg/ml used herein was 0.00862. As such, if RGe on this concentration was 0%, the population on the sampled tree could be considered sensitive and an RGe greater than 0% indicated that the population was less sensitive than the historic baseline population (Seyran et al. 2010). In the same study, for the DMI fungicide propiconazole, the probability of an isolate

sampled from the baseline population having an EC_{50} value greater than the discriminatory concentration of 1.0 µg/ml was 1.49×10^{-7} (Seyran et al. 2010). Cross resistance among the DMI fungicides is common (Köller et al. 1997) and cross resistance between propiconazole and tebuconazole is known to occur in *V. effusa*, therefore a discriminatory tebuconazole concentration of 1.0 µg/ml was appropriate (Stevenson et al. 2015). Thus, if RGr on this discriminatory concentration was 0%, the population on the sampled tree was considered sensitive to tebuconazole and an RGr greater than 0% indicated that the population was less sensitive than the historic baseline population (Seyran et al. 2010). Although a quantitative relationship between RGe or RGr values and efficacy in the field was not determined in this study, some important inferences were made, as described below.

The Super Tin 4L treated trees had consistently less leaf and nut scab than the nontreated control trees in both years of this study (Table 4.1). In 2016, mean RGe values on TPTH-amended medium for samples collected from trees treated with Super Tin 4L were between 10.9% and 40.9% (Table 4.2), and maximum leaf scab incidence, area under the disease progress curve, and maximum nut scab severity on those trees were significantly lower compared to the nontreated control trees. However, these disease measurements were significantly greater on trees treated with Super Tin 4L than on those treated with Abound, Abound plus Orius 3.6F, or Super Tin 4L plus Orius 3.6F (Table 4.1). Mean RGe values in 2017 (4.2% and 0.6%) were considerably lower than in 2016, maximum leaf scab incidence, area under the disease progress curve, and maximum nut scab severity on Super Tin 4L treated trees were significantly lower than on the nontreated control trees (Table 4.1, 4.2). Similarly, scab intensity in 2017 was

significantly lower on trees treated with Abound (maximum leaf scab incidence and area under the disease progress curve), Abound plus Orius 3.6F (maximum leaf scab incidence, area under the disease progress curve, and maximum nut scab severity), or Super Tin 4L plus Orius 3.6F (area under the disease progress curve) than on trees treated with Super Tin 4L alone (Table 4.1).

In 2017, the efficacy of Super Tin 4L in relation to the other treatments was greater than in 2016. This could be due to the lower spring rainfall observed in 2017 that resulted in less leaf scab early in the year and the higher summer rainfall that occurred after leaves were no longer susceptible. Super Tin 4L is a protectant fungicide that is generally less effective at controlling leaf scab during the early part of the season when rapid growth of susceptible leaf tissue occurs. Additionally, depending on the amount and timing of rainfall relative to fungicide applications, a considerable percentage of the fungicide may have been washed from the foliage during the early part of the 2016 growing season (Reynolds et al. 1994). Another possible explanation for greater relative efficacy of TPTH treatments in 2017 is the reduction in mean RGe values over time that resulted in a more TPTH-sensitive population in 2017. Fungicide treatment did not significantly impact sensitivity to TPTH, but sampling date did (Table 4.2). On the whole, insensitivity to TPTH in this orchard increased significantly between June and September 2016, decreased significantly between September 2016 and June 2017, and again, decreased significantly between June and September 2017 (Table 4.2). That insensitivity to TPTH decreased significantly between growing seasons, in the absence of fungicide exposure, as the pathogen overwintered, may provide evidence that insensitivity to TPTH is not stable and incurs a fitness cost to this pathogen. A similar

phenomenon was observed in the sugar beet pathogen, *Cercospora beticola*, in which insensitivity to TPTH was first identified in the U.S. in 1994 (Bugbee 1995). Results of a 10-year fungicide sensitivity monitoring program revealed that a decrease in the frequency of TPTH-insensitive isolates occurred over time when the annual number of TPTH applications was reduced from an average of 2.14 in 1998 to <1.0 from 2001 to 2008 (Secor et al. 2010). Additionally, isolates of *C. beticola* insensitive to the chemically related organotin fungicides fentin acetate and fentin chloride were found to be at a competitive disadvantage when sugar beet leaves were co-inoculated with sensitive isolates (Giannopolitis and Chrysayi-Tokousbalides 1980). Whether this is the case in *V. effusa* remains to be seen but future research is needed to further investigate the stability of TPTH insensitivity in this pathogen.

The efficacy of Orius 3.6F was greater relative to the other treatments in 2016 when compared to 2017. In 2016 mean RGr values on tebuconazole-amended medium for samples retrieved from trees treated with Orius 3.6F were between 34.6% and 45.5% (Table 4.2). On those trees, significant reductions in maximum leaf scab incidence and area under the disease progress curve were observed when compared to the nontreated control trees while maximum nut scab severity was not significantly reduced (Table 4.1). Then in 2017, mean RGr values were between 56.3% and 69.3% (Table 4.2); area under the disease progress curve was significantly reduced on trees treated with Orius 3.6F but maximum leaf scab incidence and maximum nut scab severity were not significantly different from the nontreated control trees (Table 4.1). Applications of Orius 3.6F alone did not provide significant control of leaf or nut scab, with the exception of significantly lower maximum leaf scab incidence compared to that on the nontreated trees in 2016.

This was likely due to widespread insensitivity across this orchard (Table 4.2). Similar to TPTH, fungicide treatment did not significantly impact tebuconazole insensitivity, but sampling date did (Table 4.2). Across all treatments, insensitivity to tebuconazole decreased significantly within the growing season in both years and increased significantly between years (Table 4.2). That RGr values decreased within season could be an artifact of the sampling protocol. In both years, the early-season sensitivity measurement was taken toward the end of the leaf expansion phase, as leaves began to reach maturity and became less susceptible to new infection. It is likely that more disease cycles occurred on fruit shucks and late season growth flushes after that time in both years which could have been influenced by whether new infections were coming from the same tree (autoinfection) or different ones (alloinfection) (Mundt 2009). Based on the large size of individual pecan trees, it is likely that scab within the canopy is predominantly caused by autoinfections as conidia are washed down through the canopy from the foliage above during rain events, but the dispersal of *V. effusa* inoculum between trees is not well understood. If there are relatively greater numbers of alloinfections compared with autoinfections then our RGr values may reflect the sensitivity of incoming conidia that had not experienced the same selection pressure. Whether this is the case or not, Orius 3.6F still failed to, for the most part, significantly control scab in both years of this study, providing further evidence of practical resistance to tebuconazole in this orchard.

Previous research aimed at establishing the relationship between in vitro sensitivity and efficacy has produced mixed results. Köller et al. (1997) developed the first in vitro threshold for detection of DMI resistance when they found that isolates of *V.*

inaequalis with RGr values greater than 80% on medium amended with fenarimol (0.05 µg/ml) or myclobutanil (0.1 µg/ml) were capable of causing practical field resistance. This was confirmed by conducting a greenhouse efficacy test revealing apple scab severity to be partially controlled by fenarimol or myclobutanil when leaves were inoculated with an isolate having an RGr of 88% compared to an isolate with an RGr of 42%. Miller et al. (2002) found that DMI-resistant isolates of *Sclerotinia homoeocarpa* with mean EC_{50} values 5- to 10-times greater than the baseline isolates were not effectively controlled by DMI fungicides in the greenhouse. Also investigating *S. homoeocarpa*, Popko et al. (2012) determined that isolates from a native population exhibiting RGr values greater than 50% on medium amended with propiconazole (0.1 µg/ml) were capable of causing practical resistance. Conversely, Franke et al. (1998) found no significant correlation between in vitro sensitivity of *Sclerotium rolfsii* to tebuconazole and control of southern stem rot of peanut with tebuconazole. This lack of correlation may have occurred because the isolates used in the study were sensitive enough to be adequately controlled by the labelled rate of tebuconazole. Similarly, Thomas et al. (2012) were unable to determine the relationship between fungicide sensitivity of *Stagonosporopsis citrulli* (syn. *Didymella bryoniae*) and efficacy for gummy stem blight (GSB) management because the initial population was highly resistant at the onset of the experiment. However, it was clear that if the frequency of resistance to boscalid, azoxystrobin, or thiophanate-methyl in the initial population was greater than 90%, 95%, or 80%, respectively, then these fungicides were likely to be completely ineffective against GSB.

In both years of this study, Abound and Abound plus Orius 3.6F provided superior disease control when compared with all other fungicide treatments, except Super Tin 4L plus Orius 3.6F (Fig. 4.1; Table 4.1). Azoxystrobin is the active ingredient in Abound and belongs to the QoI fungicide group known to have a high risk for resistance. That disease control was not compromised after 10 consecutive applications in both 2016 and 2017 provides practical evidence of a reduced risk of resistance predicted by the presence of an intron in the *V. effusa* cytochrome *b* gene (Standish et al. 2016). These fungicides have been used on pecan since the late 1990s and practical resistance has never been reported; however, efforts to monitor QoI sensitivity have been hindered by the lack of a reliable screening method (Seyran et al. 2010; Standish et al. 2016).

An unexpected outcome of this study was that Super Tin 4L plus Orius 3.6F provided significantly greater leaf scab control in 2016 than that of either Super Tin 4L or Orius 3.6F alone, and significantly greater nut scab control than Orius 3.6F alone (Table 4.1). To a lesser extent, this trend was also observed in 2017 with the combination resulting in significantly lower leaf scab incidence and nut scab severity than Orius 3.6F alone, and a significantly lower area under the disease progress curve than Super Tin 4L or Orius 3.6F alone (Table 4.1). Fungicides in the DMI group, like Orius 3.6F, are known to be stronger against leaf scab due to local systemic movement. However, this increased efficacy could be explained as preserving the activity of one or both fungicides as it follows an important resistance management strategy of tank-mixing an at-risk, systemic fungicide (Orius 3.6F) with a low-risk protectant (Super Tin 4L) (Köller and Wilcox 1999).

The results of this study provide insights into the relationship between efficacy of Super Tin 4L or Orius 3.6F and laboratory-based sensitivity of *V. effusa* to TPTH or tebuconazole, respectively. The active ingredients in these two fungicides are heavily relied upon by pecan producers for scab management so monitoring and managing the development of resistance to these two chemistries is of the utmost importance. The fentin hydroxide sensitivity and efficacy results reveal that RGe values between 0.6% and 40.9% could occur without causing a likely control failure, though efficacy was reduced when compared to other treatments. However, this study shows that an RGr value between 34.6% and 69.3% is likely to result in a scab control failure for trees treated with Orius 3.6F at the rate of 0.585 L/ha. In such cases, growers are advised to keep resistance management practices in mind and utilize tank mixtures to preserve fungicide activity as a means of better managing pecan scab epidemics.

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Table 4.1. Effects of bi-weekly fungicide applications on pecan scab epidemics[w]

		Leaf scab (%)				Nut scab (%)			
Year	**Treatment**	**Maximum incidence**		**AUDPC[x]**		**Maximum incidence**		**Maximum severity**	
2016[y]									
	Super Tin 4L 0.877 L/ha	51.0	b	18.9	b	100.0	a	56.9	bc
	Orius 3.6F 0.585 L/ha	43.4	b	18.3	b	100.0	a	77.4	ab
	Abound 8.77 L/ha	29.8	c	11.0	c	96.5	a	26.0	d
	Abound 0.877 L/ha + Orius 3.6F 0.585 L/ha	26.9	c	9.8	c	96.7	a	22.2	d
	Super Tin 4L 0.877 L/ha + Orius 3.6F 0.585 L/ha	33.1	c	12.0	c	100.0	a	44.2	cd
	Nontreated	61.7	a	25.7	a	100.0	a	86.1	a
2017[z]									
	Super Tin 4L 0.877 L/ha	28.8	bc	13.4	b	100.0	a	49.4	b
	Orius 3.6F 0.585 L/ha	31.1	ab	13.4	b	100.0	a	84.3	a
	Abound 8.77 L/ha	9.1	d	3.2	d	100.0	a	36.0	bc
	Abound 0.877 L/ha + Orius 3.6F 0.585 L/ha	7.5	d	2.1	d	100.0	a	25.5	c
	Super Tin 4L 0.877 L/ha + Orius 3.6F 0.585 L/ha	20.2	c	7.3	c	100.0	a	51.2	b
	Nontreated	40.9	a	18.9	a	100.0	a	97.5	a

[w]By year, means within columns followed by the same letter are not significantly different according to pairwise *t* tests of least squares means (α=0.05).
[x]Area under the disease progress curve (AUDPC) values calculated based on assessments of leaf scab incidence from 25 May to 20 July 2016 and 31 May to 28 July 2017.
[y]Applications made in 2016: 18, 28 Apr; 12, 26 May; 9, 23 Jun; 7, 21 Jul; 4, 18 Aug.
[z]Applications made in 2017: 8, 21 Apr; 5, 18 May; 1, 16, 27 Jun; 13, 27 Jul; 10 Aug.

Table 4.2. Effects of sampling date on insensitivity to fentin hydroxide and tebuconazole in *Venturia effusa* from fungicide-treated pecan trees

	Relative growth or germination (%)[w]	
Sampling date	**Fentin hydroxide[x]**	**Tebuconazole[y]**
22 Jun, 2016	10.9 (1.7) b[z]	45.5 (3.7) c
15 Sep, 2016	40.9 (6.6) a	34.6 (4.6) d
15 Jun, 2017	4.2 (0.8) c	69.3 (3.7) a
8 Sep, 2017	0.6 (0.2) d	56.3 (4.0) b
Pr > F	**<0.0001**	**<0.0001**

[w]Relative growth and germination values determined using a modified assay described by Seyran et al. (2010).

[x]Relative germination calculated as the proportion of germinated conidia on medium amended with 30 µg/ml fentin hydroxide divided by the proportion of germinated conidia on medium without fungicide, multiplied by 100; values represent the means of 30 samples collected from individual treated trees per time point with standard errors in parentheses.

[y]Relative growth calculated as the corrected mean diameter of ten micro-colonies on medium amended with 1 µg/ml tebuconazole divided the corrected mean diameter of ten micro-colonies on medium without fungicide, multiplied by 100; values represent the means of 30 samples collected from individual treated trees per time point with standard errors in parentheses.

[z]Means within columns followed by the same letter are not significantly different according to pairwise *t* tests of least squares means (α=0.05).

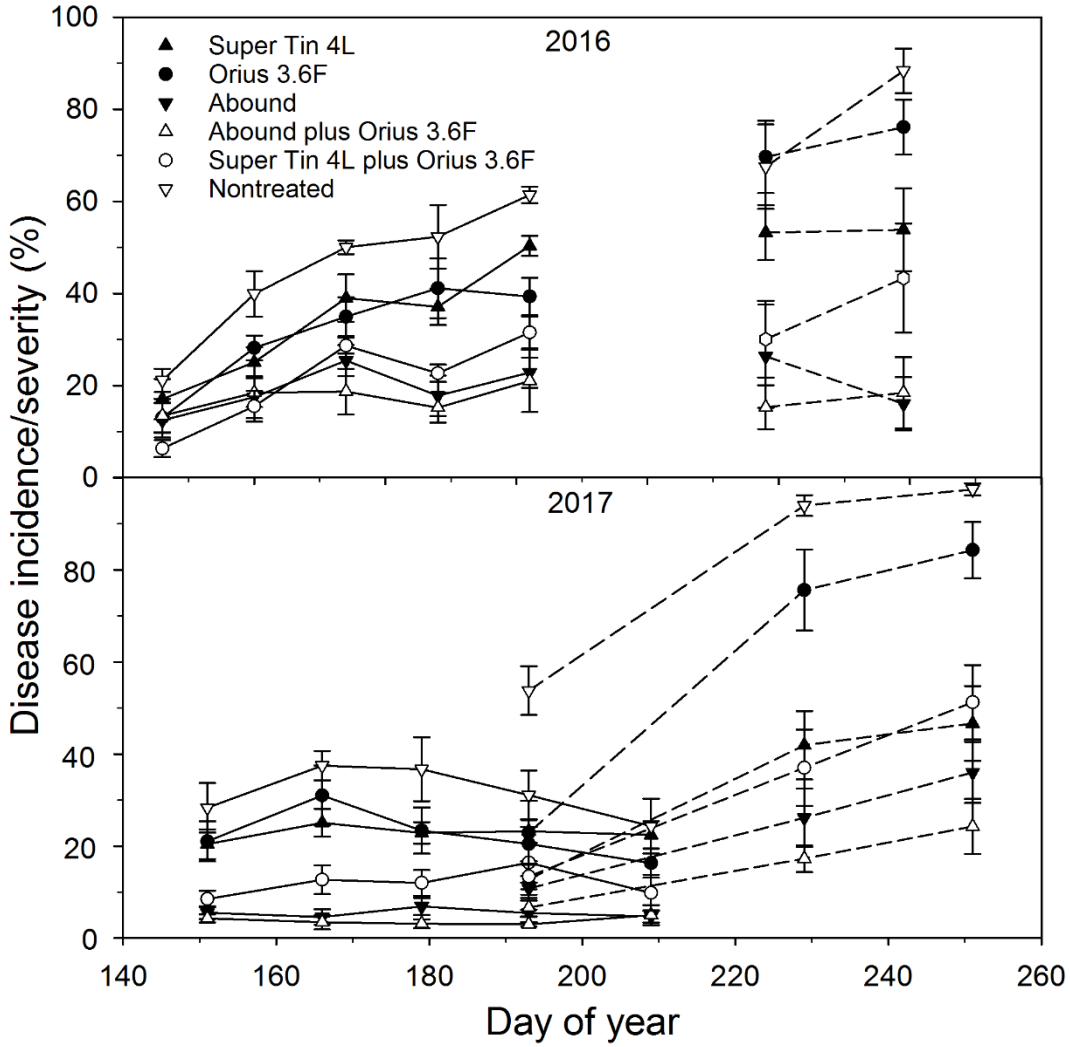

Figure 4.1. Effect of bi-weekly fungicide applications on disease progress of pecan scab epidemics in field experiments conducted in 2016 and 2017. Leaf scab incidence (indicated by the solid line) was assessed every 2 weeks after first appearance of symptoms, from 25 May to 20 July in 2016 and 31 May to 28 July in 2017; nut scab severity (indicated by the dashed line) was assessed monthly during fruit development, from 25 August to 15 September in 2016 and 12 July to 8 September in 2017.

CHAPTER 5

SPATIAL AND TEMPORAL VARIATION IN FUNGICIDE SENSITIVITY OF *VENTURIA EFFUSA* WITHIN A PECAN ORCHARD[1]

[1]Standish, J. R., Brenneman, T. B., Scherm, H., Bock, C. H., and Stevenson, K. L. 2018. To be submitted to Plant Disease.

Abstract

An 18-ha commercial pecan orchard was sampled over 3 years to study the spatial and temporal variation in fungicide sensitivity of *Venturia effusa*, cause of pecan scab. The orchard was divided into an 8×8 grid of 64 quadrats, each containing nine trees (unless there were missing trees), and samples were collected from each quadrat to be tested for sensitivity to fentin hydroxide, propiconazole, and thiophanate-methyl. Insensitivity to all three fungicides was significantly lower in 2016 compared with 2015, but significantly greater for fentin hydroxide and thiophanate-methyl in 2017. Significant spatial autocorrelation was observed in sensitivity to propiconazole in 2017 and to thiophanate-methyl in 2015 and 2017, indicating clustering, but not for any other fungicide by year combinations. Omnidirectional spatial dependency was observed for sensitivity to propiconazole and thiophanate-methyl in 2017. In both instances, the semivariance increased linearly with lag distance; however, the range of spatial dependence was greater than 276.5 m and could not be estimated accurately. Additionally, a separate sampling was conducted in all 3 years to identify an appropriate sampling size and pattern for fungicide sensitivity screening. Results showed that in most cases, a sample size of three groups of leaflets was sufficient for quantifying sensitivity of two of the three tested fungicides in this study. These results reveal that considerable biological variation in fungicide sensitivity exists orchard-scale populations of *V. effusa* and that the spatial characteristics of those populations may differ in space depending on the growing season.

Introduction

Pecan scab, caused by *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), is the major disease of pecan [*Carya illinoinensis* (Wangenh.) K. Koch] in the southeastern United States (Demaree 1924). The most practical method of managing scab is to plant scab-resistant varieties when establishing an orchard; however, *V. effusa* exhibits great genetic and pathogenic diversity that has enabled it to overcome resistance over time (Bock et al. 2014; 2017a; Conner and Stevenson 2004; Demaree and Cole 1929; Sparks 1992). Thus, successful scab management relies on frequent preventive fungicide applications made throughout the growing season (Brock and Bertrand 2007). Several fungicides are labelled for use on pecan in the U.S., the majority of which are designated as Fungicide Resistance Action Committee (FRAC) codes 1, 3, 11, 30, 33, M3, or U12; each fungicide code group has its own inherent risk of resistance (Bock et al. 2017a). Additionally, *V. effusa* is considered to have a high risk of developing fungicide resistance due to its polycyclic lifecycle, the year-round presence of scab in southeastern pecan orchards, the previously observed genetic diversity, and the widespread reliance on fungicide applications for disease management (Bock et al. 2017b; Brock and Bertrand 2007).

Complete resistance or reduced sensitivity of *V. effusa* to fungicides has been a long-standing issue beginning in the mid-1970s when control failures associated with the methyl benzimidazole carbamate (MBC) fungicide benomyl were observed in Alabama and Georgia (Littrell 1976). Later, significant reductions in sensitivity were reported to the organotin fentin hydroxide in vitro and to the demethylation inhibitors (DMIs) both in vitro and as observed control failures (Reynolds et al. 1997, Seyran et al. 2010; Standish

et al. 2018, Stevenson et al. 2004, 2015). Epidemiological studies of fungicide resistance have, for the most part, concentrated on changes in a pathogen population over time (Bauske et al. 2018; Fernández-Ortuño et al. 2017; Köller et al. 1995; Mavroeidi and Shaw 2005; Rosenzweig et al. 2008; Secor et al. 2010) but few have focused on the spatial pattern of fungicide-resistant individuals, despite the potential significance for resistance monitoring and disease management. Results of one study of the spatial pattern of dicarboximide-resistant *Monilinia fructicola* in New Zealand stone fruit orchards suggest spatial aggregation of resistant isolates in orchards (Elmer et al. 1998). Similarly, results of a study by Van der Heyden et al. (2014) provide evidence that the single-nucleotide polymorphisms related to boscalid and iprodione resistance in isolates of *Botrytis cinerea* and *B. squamosa* were spatially aggregated in grape vineyards and onion fields, respectively. In another study, Hagerty et al (2017) explored the spatial and temporal dynamics of azoxystrobin- and propiconazole-resistant isolates of *Zymoseptoria tritici* at several hierarchical levels within a winter wheat production area (region, fields within region, transects within field, samples within transect). In the same study, Hagerty et al. (2017) observed that most of the variation in fungicide sensitivity of *Z. tritici* isolates occurred among samples collected within each transect; however, they concluded that the sensitivity of those samples to propiconazole and azoxystrobin were randomly dispersed throughout transects within fields (Hagerty et al. 2017). The spatial pattern of fungicide resistance in *V. effusa* populations is not currently known, and while there is evidence of short distance dispersal (Bock et al. 2018), there is little additional information about the overall dispersal patterns of this pathogen.

In 2008, 2014, and 2015, leaf samples exhibiting symptoms of pecan scab from commercial pecan orchards in Georgia were submitted for site-specific fungicide sensitivity testing (Seyran et al. 2010; Stevenson et al. 2015). This program utilized a rapid in vitro screening method, based on single samples of 45 diseased leaflets split into three groups of 15. Conidia were collected from one lesion per leaflet and pooled together for each of the three groups. Conidial suspensions were spread across fungicide amended and non-fungicide-amended media and incubated. Depending on the fungicide being tested, conidial germination or micro-colony growth was determined and percent relative germination (RGe) or relative growth (RGr) values was calculated for each group. These three group RGe or RGr values were averaged to obtain a mean RGe or RGr value for the respective sample (Seyran et al. 2010). During the course of this monitoring program, multiple samples were submitted from spatially distinct areas within the same orchard, either on the same day or at different times throughout the growing season. In some cases, the measured level of fungicide sensitivity varied significantly among samples from the same orchard collected on the same day (K. L. Stevenson, personal communication). It is not clear whether the observed differences were due to biological variation in the pathogen population or sampling error. The sampling protocol was based on the assumption that the population of *V. effusa* within the lower canopy of an orchard block of the same cultivar, treated with the same fungicides will be uniform throughout with respect to fungicide sensitivity. If this is not the case, then quantifying the biological variability and understanding the effects of different sample sizes and patterns could prove useful for improving the accuracy and reliability of resistance monitoring efforts. Hence, the objectives of this study were to 1) investigate the spatial

variation in fungicide sensitivity of *V. effusa* to fentin hydroxide, propiconazole, and thiophanate-methyl in a commercial planting; and 2) determine an appropriate sample size and pattern for reliable estimation of fungicide sensitivity in orchard populations of *V. effusa* using the rapid in vitro method described above.

Materials and Methods

Sample collection and sample size estimation. A commercially managed 18-ha rectangular block of pecan trees with annual scab epidemics was identified in Calhoun County Georgia and consisted of approximately 576 mature pecan trees (predominantly of cv. Desirable) that were over 50 years old and between 20 and 25 m in height. Disease management consisted of fungicide applications made on a calendar-based schedule with an airblast sprayer (Appendix B, Table B.1). The trees were planted in a square pattern with approximately 18.3 m between trees in both the across- and within-row directions. A grid of 64 contiguous quadrats with roughly 54.9 m between centers was superimposed over the orchard. Each quadrat contained nine trees (Fig. 5.1A) and the grid had a maximum distance across rows and within rows of 384.1 m and a maximum data distance of 543.1 m (the diagonal distance between grid corners). Approximately six leaflets exhibiting typical scab symptoms were collected from the lower canopy of each tree (≤8 m) within each quadrat on 22 June 2015, 20 June 2016, and 10 July 2017. All leaflets from each quadrat were combined, placed into a plastic bag, and refrigerated (4ºC) until fungicide sensitivity testing could be conducted, generally within 7 days. Following this sampling strategy, leaflets from each quadrat were treated as one sample for fungicide sensitivity testing using the previously mentioned rapid in vitro assay developed by

Seyran et al. (2010), as described below. Additionally, 225 leaflets exhibiting typical scab symptoms were collected from the same orchard block described above following three systematic sampling patterns (Fig. 5.1B): a "W" pattern (all 3 years); "X" and diamond "◇" patterns (2016 and 2017). The sampled leaflets were combined and randomly divided into 15 groups of 15 leaflets to be screened separately for sensitivity to propiconazole, fentin hydroxide, and thiophanate-methyl, using the fungicide concentrations and methods described below.

Fungicide sensitivity. Technical-grade propiconazole and fentin hydroxide (99.5 and 98.7% a.i., respectively; Chem Service, Inc., West Chester, PA) were each dissolved in acetone to concentrations of 1,000 µg/ml and 30,000 µg/ml, respectively; and thiophanate-methyl (99.5% a.i.; Chem Service, Inc.) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 5,000 µg/ml. To obtain final concentrations of fentin hydroxide (30 µg/ml) and thiophanate-methyl (5 µg/ml), 1 ml of the respective solution was added to 1 liter of autoclaved 2% water agar (WA; 20 g Bacto agar per liter of water) that had cooled to 50°C. Similarly, for propiconazole, a final concentration of 1 µg/ml was obtained by adding 1 ml of the propiconazole solution to 1 liter of autoclaved and cooled quarter-strength potato dextrose agar (qPDA; 9.75 g PDA and 11.25 g Bacto agar per liter of water). A non-fungicide-amended control containing 1 ml/liter of either acetone or DMSO was included for each fungicide (Seyran et al. 2010). The media were poured into petri dishes (15 × 100 mm diameter) and refrigerated in the dark at 4ºC for use within 7 days.

A sample-based bulk-conidia assay described by Seyran et al. (2010), with slight modifications, was used for sensitivity testing and is summarized here. The samples

from each quadrat were tested separately and were divided into three groups of 15 individual lesions for a total of 45 tested lesions per quadrat sample. Conidia were dislodged and collected by gently pumping 9 µl of a sterile deionized water solution amended with antibiotics and Tween 20 (50 µg/ml each of chloramphenicol, streptomycin sulfate, and tetracycline, and one drop Tween 20 per 100 ml) onto the surface of each lesion (Seyran et al. 2010). Conidia from each group of 15 lesions were collected separately in a 2.0-ml microcentrifuge tube and mixed using a vortex mixer (Seyran et al. 2010).

For fentin hydroxide and thiophanate-methyl, 23 µl of each of the three conidial suspensions per sample were placed onto two replicate dishes of fentin hydroxide-amended medium, thiophanate-methyl-amended medium, and the respective non-amended WA control plates for both (Seyran et al. 2010). Conidia were incubated at room temperature (23 to 25ºC) for 48 h in the dark, and conidial germination was estimated for 50 conidia per replicate. A conidium was considered germinated if the germ tube was at least four times as long as the diameter of the conidium. For each of the three groups, percent relative germination (RGe) was calculated as the proportion of germinated conidia on fungicide-amended medium divided by the proportion of germinated conidia on non-amended medium, multiplied by 100. These three mean RGe values were averaged to yield a mean RGe for each sample (Seyran et al. 2010).

For propiconazole, 23 µl of each of the three conidial suspensions per sample were placed onto two replicate dishes of propiconazole-amended and non-amended qPDA (Seyran et al. 2010). Conidia were spread using a sterile glass rod and similarly incubated at room temperature (23 to 25ºC) in the dark. After 72 h of incubation, a

compound microscope (×100) with an ocular micrometer was used to measure the maximum colony diameters of ten single and well-separated colonies (Seyran et al. 2010). To correct for the average combined length of a germinated conidium and germ tube, 15 µm was subtracted from all micro-colony diameter measurements. For each of the three groups, percent relative growth (RGr) was calculated as the corrected mean diameter of ten micro-colonies on fungicide-amended medium divided by the corrected mean diameter of ten micro-colonies on non-amended medium, multiplied by 100. The three RGr values were averaged to calculate the mean RGr for each sample (Seyran et al. 2010).

Data analyses. Based on values from the 64 quadrats, RGe or RGr for each of the three fungicides in this orchard were compared over the 3 years with the nonparametric Kruskal-Wallis test performed using the NPAR1WAY procedure of SAS (v. 9.4, SAS Institute, Cary, NC). Means separation was conducted on ranked values in PROC GLM. Similarly, the non-parametric Wilcoxon signed rank test was performed in PROC UNIVARIATE to determine whether differences in RGe or RGr values in individual quadrats from one year to the next were significantly different from zero; the analysis was performed for each respective fungicide. Residual and quantile plots were examined to assess normality of RGe or RGr values for each of the fungicide by year combinations, as well as for the associated differences in RGe or RGr values between years for each fungicide. If distributions were significantly different from normal, then non-parametric statistical methods were used, as described above.

Initially, maps of relative fungicide sensitivity values were produced for each fungicide by year combination by assigning the RGe or RGr values to the centers of their

respective 64 spatially referenced quadrats. Further analyses were conducted on ln-transformed RGe or RGr values for each fungicide by year combination using the VARIOGRAM and REG procedures in SAS. Geary's *C* was estimated to indicate the presence of positive or negative spatial autocorrelation (Geary 1954) and a standardized Z statistic was calculated using the variance and expected values of Geary's *C* to determine whether the observed value was significantly different from the expected value of 1.0 (Geary 1954). A semivariance analysis was conducted to characterize the spatial dependency of ln-transformed RGe or RGr values. Omnidirectional experimental semivariograms were constructed for each fungicide by year combination by plotting semivariances as a function of lag distance. The semivariance was calculated for each of nine equi-frequent distance classes (54.9, 77.6, 109.7, 122.7, 166.5, 197.8, 223.8, 241.0, and 276.5 m), each with at least 95 pairs of observations.

For sample size estimation, the mean RGe or RGr value was calculated using the ALLCOMB function in SAS for all possible combinations of a sample size of two and up to a sample size of 14 groups of leaflets. The sample mean for each individual combination of samples was calculated and then the variance of those sample means for each sample size was calculated. The coefficient of variation (C.V.) was calculated as the standard deviation of the mean sample variance for all combinations per sample size divided by the mean RGe or RGr value for those combinations. The coefficient of variation was used to estimate the reliability of the currently used sample size (three groups of 15 leaflets) in tests of fungicide sensitivity using the rapid assay.

Results

For all three of the tested fungicides, sensitivity within the orchard varied significantly among years (Table 5.1). For fentin hydroxide, mean RGe values in quadrats ranged from 0.0 to 54.5% in 2015; 2.5 to 40.5% in 2016; and 0.0 to 61.9% in 2017. Mean RGr values for propiconazole ranged from 0.0 to 149.2% in 2015; 0.3 to 128.8% in 2016; and 0.0 to 97.1% in 2017. For thiophanate-methyl, the mean RGe values ranged from 0.8 to 115.4% in 2015; 14.4 to 107.9% in 2016; and 0.0 to 151.2% in 2017. All RGe values for fentin hydroxide were non-normal, as were the differences among years. The thiophanate-methyl RGe values were normally distributed in 2015 and 2016 but not in 2017, and the differences among years were normally distributed except for the change from 2016 to 2017. Similarly, RGr values for propiconazole were normally distributed in 2016 and 2017 but not in 2015; the differences among years were normally distributed except for the change from 2015 to 2017. For all three fungicides, RGe or RGr values were significantly greater in 2016 than in 2015 but RGe values for thiophanate-methyl and fentin hydroxide were significantly lower in 2017 compared with 2015 and 2016. The mean percent change in fentin hydroxide RGe values for samples from individual quadrats followed a similar pattern to the mean RGe values from the orchard overall. The changes from 2015 to 2017 were not significant, but the changes between individual years were significant with RGe values increasing from 2015 to 2016 and decreasing from 2016 to 2017 (Appendix B, Table B.2). Similarly, propiconazole RGr values for individual quadrats followed the same pattern as the mean values in the orchard, increasing significantly from 2015 to 2016 and from 2015 to 2017, but not from 2016 to 2017. For thiophanate-methyl, the RGe values of individual quadrats again

followed the same pattern as the orchard values, with a significant increase from 2015 to 2016 and a significant decrease from 2016 to 2017. However, unlike the overall orchard values, the decrease from 2015 to 2017 was not significant ($P = 0.1136$).

Sensitivity to fentin hydroxide, thiophanate-methyl, and propiconazole varied greatly among quadrats within years (Fig. 5.2), with some evidence for spatial autocorrelation and clustering. The observed values of Geary's *C* were significantly less than the expected values for propiconazole RGr values in 2017 and for thiophanate-methyl RGe values in 2015 and 2017, providing evidence of spatial autocorrelation (Table 5.2). There was no evidence of spatial autocorrelation in fentin hydroxide RGe values among quadrats in any of the 3 years. The semivariograms revealed two patterns: 1) no quantitative relationship between semivariance and lag distance, or 2) spatial dependence indicated by a linear increase in semivariance values with distance between quadrats (Table 5.2). The first pattern was observed in most cases including fentin hydroxide RGe values in all 3 years, propiconazole RGr values in 2015 and 2016, and thiophanate-methyl RGe values in 2015 and 2016. The second pattern was observed for both propiconazole RGr values and thiophanate-methyl RGe values in 2017 (Table 5.2). In both the case of propiconazole RGr values and thiophanate-methyl RGe values in 2017, the semivariance increased linearly with lag distance indicating that the range of spatial dependence was greater than 276.5 m, but could not be accurately estimated with the current data set.

Regardless of the sampling pattern used, for two of the three tested fungicides a sample size of three groups of 15 leaflets (referred to here as samples) resulted in C.V.s that were close to the threshold of 0.2 and additional precision could be gained by adding

a fourth group. In some cases, the total number of samples tested was less than expected due to contamination in the petri dishes which prevented an accurate measure of micro-colony growth (propiconazole) or conidial germination (fentin hydroxide). Nevertheless, seven sampling pattern by year combinations were examined, the results of which are presented in Table 5.3. For fentin hydroxide, three samples resulted in a range of C.V. values between 0.19 and 1.33. In 2016, three samples were very reliable with C.V. values between 0.19 and 0.29, but the mean RGe values for all three sampling patterns were considerably greater that year than in 2015 or 2017. Similarly, for propiconazole, three samples had a range of C.V. values between 0.10 and 0.68. Once again, a lower C.V. was observed with three samples when mean RGr values were higher. The RGe values for thiophanate-methyl were far more consistent over years compared with those of fentin hydroxide or the RGr values of propiconazole (Table 5.3). Three samples provided a range of C.V. values between 0.16 and 0.39 for thiophanate-methyl RGe values.

Discussion

Monitoring and managing the development of fungicide resistance in *V. effusa* is important to preserve fungicide efficacy as well as to help guide fungicide use. The results of a recent fungicide monitoring program revealed unexpected variation in the measured levels of sensitivity among samples collected from the same orchard on the same day (K. L. Stevenson, personal communication). The results presented here reveal considerable spatial and temporal variation in fungicide sensitivity across a commercial

orchard block in addition to providing evidence that the reliability of the established sample size will vary greatly based on the specific fungicide tested and year of collection.

Sensitivity to each of the three fungicides tested varied significantly over the 3 years of this study. The RGe and RGr values for all three fungicides increased from 2015 to 2016 but decreased from 2016 to 2017 for thiophanate-methyl and fentin hydroxide. The change in individual quadrat sensitivity values, for the most part, followed a similar trend to the mean sensitivity of the samples collected in this orchard. The significant decrease in insensitivity to fentin hydroxide and thiophanate-methyl observed from 2016 to 2017 could be the result of natural variation in the population, a decrease in the use of either active ingredient reducing selection on the pathogen, and/or fitness penalties associated with insensitivity. In the case of thiophanate-methyl, a fitness penalty is not likely as resistance to MBC fungicides has been shown to be stable over decades with resistant and sensitive isolates appearing equally fit (Campbell 1998; Jones and Ehret 1976). Likewise, cross-resistance within this class is frequently observed (Brent and Hollomon 2007) and thiophanate-methyl use has increased across Georgia pecan orchards since 2008 (Stevenson et al. 2015). Although the RGe values observed in this study fluctuated significantly from one year to the next, they were consistent with those obtained in other orchards across Georgia (Stevenson et al. 2015). With fentin hydroxide insensitivity, the observed increase and decrease, from 2015 to 2016 and 2016 to 2017, in the absence of fungicide while the pathogen overwintered may provide evidence that insensitivity is not stable and incurs a fitness cost to the pathogen. Isolates of the sugar beet pathogen, *Cercospora beticola*, insensitive to the chemically related fungicides fentin acetate and fentin chloride were found to be at a competitive disadvantage when

co-inoculated onto sugar beet leaves with sensitive isolates (Giannopolitis and Chrysayi-Tokousbalides 1980). Similarly, the frequency of insensitive *C. beticola* isolates decreased over a 10-year period when the annual number of fentin hydroxide applications was reduced from an average of 2.14 in 1998 to <1.0 from 2001 to 2008 (Secor et al. 2010). A similar phenomenon was observed in *V. effusa* where RGe values for fentin hydroxide decreased significantly between growing seasons, in the absence of fungicide exposure (Standish et al. 2018). Sensitivity testing in that study was not based on monoconidial isolates, but on bulk conidia samples tested using the same rapid method and fungicide concentration (30 µg/ml) as in the present study. Future studies investigating the stability and mechanisms of fentin hydroxide insensitivity in *V. effusa* are warranted to better understand the variation observed here and in previous studies.

The temporal and spatial variation in fungicide insensitivity observed in this study may be partially explained by pathogen dispersal patterns. Samples were collected from the lower canopy (<8 m) of the trees in each quadrat but because the trees sampled in this study were >20 m tall, there was a considerable portion of these trees that was not sampled. Likewise, due to the height of pecan trees, obtaining complete spray coverage when applying fungicides with a large air-blast sprayer is a concern for growers and researchers alike. Previous studies have shown that large ground-based orchard air-blast sprayers are capable of providing good disease control at heights of up to 12 m, at which point the spray coverage declines significantly (Bock et al. 2013; 2015). Thus with an assumption of poor spray coverage above 12 m, conidia on lesions in the tops of the trees are unlikely to be exposed to the same selection as those existing in the lower canopy. The dispersal of propagules of *V. effusa* throughout an orchard is not well understood;

conidia have been observed to disperse during daylight hours by wind and rain splash (Gottwald and Bertrand 1982), and the results of recent studies into the genetic diversity of the pathogen suggest that short distance events, typical of splash dispersal, do occur (Bock et al. 2018). Based on the large size of individual pecan trees, it is likely that scab lesions within the canopy are caused by autoinfections resulting from splash dispersed conidia from the foliage above during rain events, but the relative contributions of wind- or splash-dispersed conidia to scab epidemics are not known. If lower canopy infections are caused predominantly from conidia produced by lesions in the upper canopy, then the RGe and RGr values observed in the lower canopy may be lower than expected due to a dilution effect. Whether this is the case or not remains to be seen as the vertical distribution of fungicide-resistant individuals has not been explored. However, such a study could provide valuable information on the epidemiology of fungicide-resistant *V. effusa*.

The results of this study show that in 2017, the populations at the quadrat scale were spatially dependent based on relative sensitivity to thiophanate-methyl and propiconazole. Additionally, spatial autocorrelation was observed in three of the nine fungicide by year combinations. The presence of spatial dependence could provide evidence of alloinfection from wind-dispersed conidia that resulted in large clusters of disease dominated by propiconazole and thiophanate-methyl insensitive phenotypes. At present, there is little evidence of clonality existing beyond the scale of tens of centimeters (Bock et al. 2018) and thus, propagation of these insensitive phenotypes may occur regardless of genotype. The fact that spatial dependence was observed in 2017 and in no other year could also be the result of high wind events associated with several

tornadoes moving through the region, and this orchard, between the 2016 and 2017 growing seasons. Approximately 10% of the trees in this test site were lost as a result (Fig. 5.1A), and during those storms, conidia were likely to have been widely dispersed throughout the orchard. However, it is important to note that for these two cases the range of spatial dependence could not be estimated but is likely greater than 276.5 m suggesting the presence of relatively large clusters of trees infected with *V. effusa* with similar levels of sensitivity to fungicides. The overall size of individual pecan trees and the distance between trees (approximately 18 m both within and across rows) required that a relatively large geographic area be sampled and led to our decision to include nine trees within each of the 64 quadrats. As a result, the distance between individual quadrats was approximately 54 m, which may have been too great a distance to characterize the specific range of spatial dependence accurately.

Few epidemiological studies of fungicide resistance have focused on the spatial pattern of fungicide-resistant individuals. Elmer et al. (1998) used Lloyd's index of patchiness to detect aggregation in dicarboximide-resistant isolates of *M. fructicola* across stone-fruit orchards in a 3-year study. They observed spatial autocorrelation in two of six tested orchards in each of the three sampled years. The six orchards were divided into 100 contiguous quadrats containing either one, two, or three trees each, compared with the current study where quadrats contained nine pecan trees. The six orchards were divided into 100 contiguous quadrats containing either one, two, or three trees each, with approximately 1 to 5.5 m between trees within rows and 3.5 to 5.5 m across rows compared with the current study where quadrats contained nine pecan trees with 18.3 m between trees in both directions. In the present study, smaller quadrats with

fewer trees may have allowed for more precision in determining spatial variation across this orchard. However, quadrat size was chosen based on the relatively large size of individual pecan trees, the total number of quadrats, our ability to process a fixed number of samples, and the likelihood that conidia may be dispersed among trees by wind. The spatial correlations of resistant *M. fructicola* were not significant beyond one quadrat indicating that resistant isolates did not spread far beyond their original focus and lacked the ability to remain in or dominate the field population. In the current study, the two observed cases of spatial dependency (sensitivity to propiconazole and thiophanate-methyl in 2017) were characterized by a range of greater than 276.5 m, which implies that resistant isolates of *V. effusa* may disperse readily. Additionally, the observed RGe or RGr values over 3 years imply that these resistant isolates are fit enough to remain in field populations. As previously mentioned, MBC-resistant isolates of other fungi appear to be equally as fit as their sensitive counterparts (Campbell 1998; Jones and Ehret 1976); likewise, there is evidence that DMI-resistance is a phenotypically stable trait in *V. effusa* (Standish et al. 2018). In another study, Van der Heyden et al. (2014) observed spatial autocorrelation in the frequency of single nucleotide polymorphisms (SNPs) related to fungicide resistance in *B. cinerea* and *B. squamosa* in grape vineyards and onion fields, respectively. In that same study, various levels of anisotropy were detected, and a directional bias was observed; autocorrelation was characterized by a longer range in one direction that corresponded with hedged rows. Directional semivariograms are not presented here because there was only a limited number of data points in the across- and within-row directions. Moreover, the trees sampled in this study were large enough to reach canopy closure in both directions making a directional bias unlikely. Additionally,

semivariograms for most of the fungicide by year combinations analyzed herein provided no evidence of spatial dependency. Similar results were observed in a study by Hagerty et al. (2017), in which azoxystrobin- and propiconazole-resistant phenotypes of *Z. tritici* were randomly distributed within sampled fields; however, their smallest sampling scale was a 30-m transect within a field, and fields were not divided into quadrats.

In the current study, we assessed the reliability of the assay based on the number of samples used for sensitivity testing as well as the general sampling pattern used to collect those leaflets. The reliability of sensitivity testing based on three samples differed considerably based on fungicide tested and year of sampling. For individual fungicides, the sampling patterns differed slightly in terms of the C.V. attained with three samples but not enough to select one pattern over the others. The rapid in vitro screening method used here for evaluating fungicide sensitivity utilized concentrations of fentin hydroxide, thiophanate-methyl, and propiconazole that were described previously (Seyran et al. 2010). Discriminatory concentrations of 30 µg/ml fentin hydroxide, 1 µg/ml thiophanate-methyl, and 1 µg/ml propiconazole were chosen, with RGe or RGr values of 0% considered sensitive and values greater than 0% indicated that the tested population was less sensitive than the historic baseline population (Seyran et al. 2010). Based on preliminary studies, a concentration of 5 µg/ml thiophanate-methyl was better at discriminating between samples and was chosen in place of that which was used previously (Seyran et al. 2010). As *V. effusa* is a very slow-growing organism, traditional in vitro bioassays require approximately 6 weeks of incubation post-isolation to allow for adequate mycelial growth and then an additional 4 to 5 weeks for mycelial growth assays (Reynolds et al. 1997). Conversely, the rapid method described by Seyran et al. (2010)

allows for a 3-day turnaround on sensitivity testing and relies upon a single sampling of 45 diseased leaflets that are divided into three groups of 15 (referred to here as samples). While not directly comparable, the results of this sampling study show that all three sampling patterns performed similarly for propiconazole in 2017 and thiophanate-methyl in 2015 and 2017, corresponding to cases in which spatial autocorrelation was identified in the greater orchard sampling.

The three active ingredients tested in this study are each key components in scab management, thus, preserving their efficacy through sensitivity monitoring and resistance management is an important endeavor. The results of this study reveal considerable biological variation in fungicide sensitivity in populations of *V. effusa* of the commercial orchard that was sampled and provide insight into the spatial and temporal dynamics of fungicide sensitivity in this important pathogen of pecan. While RGe and/or RGr values for the three fungicides differed significantly by year, there was limited evidence of spatial dependency; in fact, in most fungicide by year combinations, dependency was not observed. Additionally, we provide quantitative evidence of the reliability of the sensitivity screening technique described by Seyran et al. (2010). Regardless of sampling pattern, three groups of 15 leaflets, in most cases, will allow for the accurate quantification of sensitivity to two of the three fungicides tested in this study. By including more groups, the reliability of the assay increases; however, including more samples may not always be practical due to the time and materials required for sensitivity testing. These results underscore the importance of collecting a representative sample to better characterize the fungicide sensitivity in the orchard of interest (i.e., sampling a large area and not individual scab foci within the orchard). In such a case, the fungicide

sensitivity measurements would be more accurate and would more reliably reflect the true situation in the orchard. Informed decisions on future fungicide applications or resistance management strategies could then be made to better manage annual epidemics of pecan scab.

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Table 5.1. Relative insensitivity to fentin hydroxide, propiconazole, and thiophanate-methyl in *Venturia effusa* collected from a commercial pecan orchard in 2015, 2016, and 2017

	Relative growth or germination (%)[w]		
Year	**Fentin hydroxide**[x]	**Propiconazole**[y]	**Thiophanate-methyl**[x]
2015	6.7 (12.4) b[z]	15.0 (34.4) b	44.5 (23.8) b
2016	12.7 (7.5) a	46.7 (25.5) a	56.7 (20.7) a
2017	7.0 (13.5) b	51.3 (22.4) a	36.1 (32.4) c
Pr > χ^2	**<0.0001**	**<0.0001**	**<0.0001**

[w]Relative growth and germination values determined using a modified assay described by Seyran et al. (2010). Values represent the means of 64 samples collected from groups of nine trees with standard deviation in parentheses.
[x]Relative germination calculated as the proportion of germinated conidia on medium amended with 30 µg/ml fentin hydroxide or 5 µg/ml thiophanate-methyl divided by the proportion of germinated conidia on medium without fungicide, multiplied by 100.
[y]Relative growth calculated as the corrected mean diameter of ten micro-colonies on medium amended with 1 µg/ml propiconazole divided the corrected mean diameter of ten micro-colonies on medium without fungicide, multiplied by 100.
[z]Means within columns followed by the same letter are not significantly different according to the non-parametric Kruskall-Wallis test (α=0.05).

Table 5.2. Semivariogram characteristics, model parameters, and autocorrelation statistics for the distribution of relative germination/growth values of *Venturia effusa* samples from 2015 to 2017

		Spatial autocorrelation[a]		Semivariogram[b]			
Fungicide	**Year**	**Geary's *C***	***P* value**	**Model**	**R^2**	**Intercept**	**Slope**
Fentin hydroxide	2015	0.998	0.9336	Nugget	-	-	-
	2016	1.000	0.9865	Nugget	-	-	-
	2017	0.948	0.0523	Nugget	-	-	-
Propiconazole	2015	1.046	0.0905	Nugget	-	-	-
	2016	1.031	0.2449	Nugget	-	-	-
	2017	0.826	<0.0001	Linear	0.63	0.3613	0.0011
Thiophanate-methyl	2015	0.945	0.0423	Nugget	-	-	-
	2016	0.950	0.0647	Nugget	-	-	-
	2017	0.932	0.0117	Linear	0.82	1.093	0.0013

[a]Spatial autocorrelation assessed using Geary's *C*. A standardized Z statistic was calculated to determine whether the observed value was significantly different from the expected value of 1.0.

[b]Description of variation for the semivariogram of each individual fungicide by year combination. Model refers to the regression model that best fit the semivariogram. Nugget implies a "pure nugget effect" which characterizes either spatial variation at scales smaller than the distance between sampling units or variability from measurement errors.

Table 5.3. Descriptive statistics and estimated reliability of fungicide sensitivity testing for all possible combinations of three groups of 15 leaflets

Fungicide[a]	Year	Pattern	n[b]	Mean[c]	Stdev	CV[d]
Fentin hydroxide	2015	W	15	3.5	7.1	1.03
	2016	W	15	21.4	8.7	0.21
		◇	15	26.9	15.3	0.29
		X	15	22.3	8.1	0.19
	2017	W	12	1.7	4.4	1.33
		◇	12	2.3	4.1	0.90
		X	12	0.6	1.2	0.94
Propiconazole	2015	W	14	28.9	38.2	0.68
	2016	W	15	14.7	17.7	0.62
		◇	15	40.2	12.4	0.16
		X	14	40.5	23.2	0.29
	2017	W	14	80.2	15.3	0.10
		◇	10	63.4	15.5	0.12
		X	12	54.7	35.6	0.33
Thiophanate-methyl	2015	W	15	86.4	24.4	0.16
	2016	W	15	54.0	17.1	0.18
		◇	15	35.5	16.7	0.24
		X	15	38.9	13.6	0.18
	2017	W	15	62.7	30.2	0.25
		◇	15	45.6	34.2	0.39
		X	15	46.9	21.8	0.24

[a]Relative germination (RGe) was determined on media amended with 30 µg/ml fentin hydroxide or 5 µg/ml thiophanate-methyl; relative growth (RGr) was determined on media amended with 1 µg/ml propiconazole

[b]Number of groups of 15 leaflets included in the sample size estimates for each respective shape, fungicide, and year.

[c]The average of the mean RGe or RGr values for each combination of groups of 15 leaflets.

[d]Coefficient of variation for all combinations of three groups of 15 leaflets. Coefficient of variation expressed as (standard deviation of the mean sample variance for all possible combinations per sample size)/(mean of RGe or RGr values for those combinations).

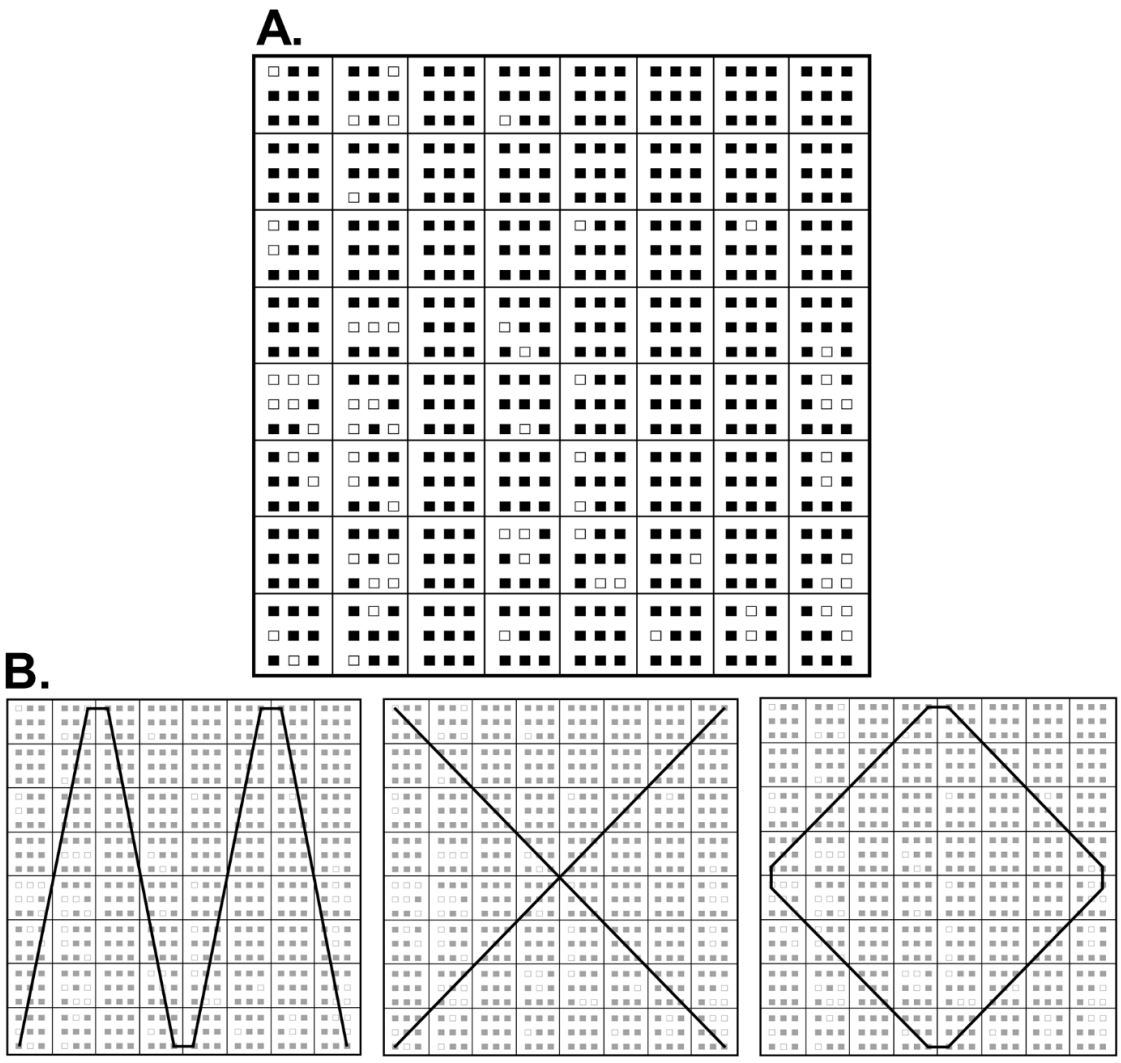

Figure 5.1. A. Schematic representation of the commercial orchard sampled in each year of this study. The solid boxes represent pecan trees in the orchard divided into quadrats while the open boxes represent trees lost to tornado damage in the fall of 2016. **B.** Systematic sampling patterns used to collect leaflets for estimating appropriate sample sizes for fungicide sensitivity testing.

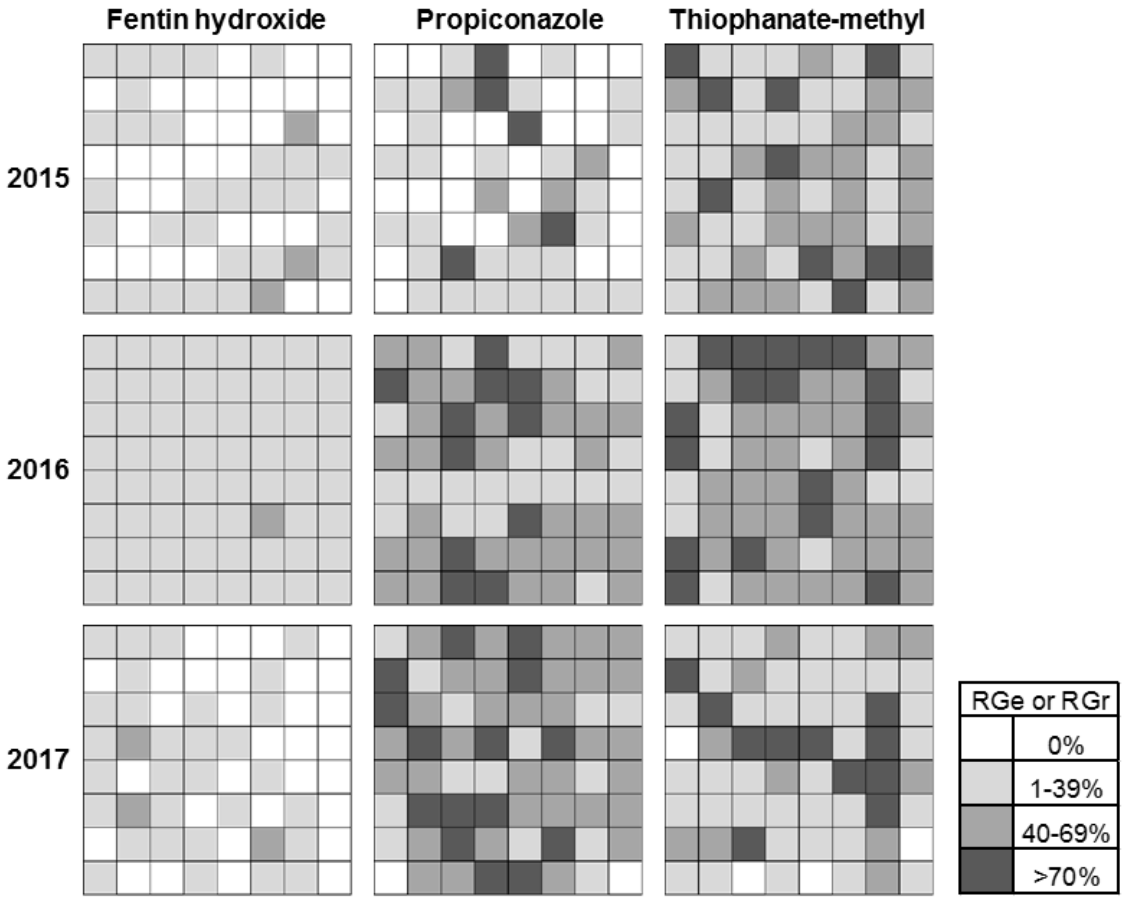

Figure 5.2. Categorized relative fungicide insensitivity of *Venturia effusa* collected from a pecan orchard divided into 64 quadrats, measured as percent relative germination (RGe) of conidia on medium containing fentin hydroxide (30 µg/ml) or thiophanate-methyl (5.0 µg/ml), or percent relative growth (RGr) of micro-colonies grown on medium containing propiconazole (1.0 µg/ml). Each quadrat contained nine trees.

CHAPTER 6

ASSESSING FITNESS COSTS AND PHENOTYPIC INSTABILITY OF FENTIN HYDROXIDE AND TEBUCONAZOLE RESISTANCE IN *VENTURIA EFFUSA*[1]

[1]Standish, J. R., Brenneman, T. B., Brewer, M. T., and Stevenson, K. L. 2018. To be submitted to Plant Disease.

Abstract

Sensitivity monitoring of *Venturia effusa*, cause of pecan scab, has revealed insensitivity to fentin hydroxide and tebuconazole, but recent research indicates that the insensitivity to fentin hydroxide is not stable. A study was undertaken to determine whether a fitness cost may be responsible for this instability. Experiments were conducted to evaluate fitness components and phenotypic stability of insensitivity of *V. effusa* to fentin hydroxide and tebuconazole. Conidial production, conidial germination, micro-colony growth, sensitivity to osmotic stress, and sensitivity to oxidative stress were compared for isolates with differing sensitivities to both fungicides. Percent conidial germination decreased linearly with increasing fentin hydroxide insensitivity, and micro-colony growth on 1.0 mM H_2O_2 decreased linearly with increasing tebuconazole insensitivity. Stability of resistance was assessed on concentrations of 1.0, 3.0, and 10 µg/ml of both fungicides prior to, and after five transfers on non-fungicide amended medium. Tebuconazole insensitivity was stable after transfers, but fentin hydroxide insensitivity on 1.0 and 3.0 µg/ml decreased significantly after transfers, indicating instability. Here we provide evidence that tebuconazole insensitivity is stable and that fentin hydroxide insensitivity is not in *V. effusa*. These results suggest that fentin-hydroxide-resistant *V. effusa* isolates have reduced conidial viability, and may allow the population to regain sensitivity in the absence of this important fungicide.

Introduction

In the southeastern United States, the successful management of pecan scab, caused by *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), may be achieved through the use of preventive fungicide applications made from April through August (Brock and Bertrand 2007a). Pecan producers are advised to apply fungicides using either a calendar-based schedule that may result in 10 or more applications per season, or a weather-based method that, in a dry season, may require fewer applications compared with the calendar-based method (Brenneman et al. 1998; Brock and Bertrand 2007b). Fungicides belonging to several different chemical groups are approved for use on pecan in the U.S. and include Fungicide Resistance Action Committee (FRAC) codes 1, 3, 11, 30, 33, M3, and U12, representing the methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), organotin compounds, phosphonates, dithiocarbamates, and guanidines, respectively (Bock et al. 2017). The inherent risk of resistance developing in *V. effusa* to the active ingredients within each of these fungicide groups ranges from "low to medium" to "high" and is magnified by the inability to provide uniform coverage in larger pecan trees (Bertrand and Brenneman 2001; Bock et al. 2013; FRAC 2018). Similarly, *V. effusa* survives as stromata within the canopy of pecan trees from season to season, which combined with the potential for sexual recombination, leads to a high risk for resistance to develop in this pathogen (Demaree 1924; FRAC 2013; Young et al. 2018).

The results of a recent grower-funded sensitivity monitoring program revealed that the scab pathogen is gradually becoming less sensitive to many important fungicides,

specifically the organotin fungicide fentin hydroxide and the DMIs, and that the levels of insensitivity were consistent with fungicide use patterns across southwest Georgia (Seyran et al. 2010; Stevenson et al. 2015). Fitness, or the ability of an organism to survive and reproduce successfully, plays a major role in the development and phenotypic stability of fungicide resistance (Cox et al. 2007, Ishii 2015, Pringle and Taylor 2002). If resistant isolates are less fit than sensitive isolates, the frequency of resistant isolates in the population will decrease in the absence of the fungicide, and resistance will not be stable (Mikaberidze and McDonald 2015). Whether resistant isolates show a reduction in fitness appears to be dependent on the pathogen and the mode of action of the fungicide (Hollomon 2015). Nevertheless, if resistant isolates are less fit, the portion of the pathogen population consisting of resistant isolates will decline in the absence of fungicide selection (Ishii 2015). Over time, this could result in a mostly sensitive population and the re-establishment of the efficacy of the fungicide in question (Ishii 2015). Whether isolates of *V. effusa* insensitive to fentin hydroxide or the DMI fungicides are as fit as their sensitive counterparts in the absence of the fungicides remains to be determined.

Pecan producers throughout the southeastern U.S. rely on the commercial products containing fentin hydroxide or the DMIs for effective scab management. Thus, the objectives of this study were to 1) evaluate fitness in isolates of *V. effusa* with differing levels of sensitivity to fentin hydroxide or tebuconazole, and 2) investigate the stability of insensitivity to fentin hydroxide or tebuconazole over several successive successive hyphal transfers.

Materials and Methods

Fungal isolates and fungicide sensitivity testing. A total of 20 monoconidial isolates of *V. effusa* collected from Georgia pecan orchards were investigated in this study (Table 6.1). The isolates were grown on potato dextrose agar amended with tetracycline (50 mg/liter), streptomycin sulfate (50 mg/liter), and chloramphenicol (50 mg/liter) (PDA+) in petri dishes (15 × 100 mm diameter). After 4 to 6 weeks, five 6-mm diameter mycelial plugs from each respective *V. effusa* colony were removed and placed into a microcentrifuge tube containing 1 ml sterile potato dextrose broth (PDB) and three sterile 3-mm glass beads. Fungal material was macerated using a bead beating apparatus (Mini-Bead Beater, Biospec, Bartlesville, OK) for 1 min before being transferred to a tube containing 5 ml PDB and mixed using a vortex mixer. This fungal slurry was pipetted in aliquants of 0.6 ml and spread across petri dishes (15 × 60 mm diameter) containing oatmeal agar (30 g ground oats [Quaker Oats Company, Chicago, IL] and 20 g agar per liter of water) amended with the previously mentioned antibiotics (OA+) (Turechek and Stevenson 1998). Dishes were incubated under a 14-h day/night cycle with fluorescent light sources at 25ºC for 14 days, when the cultures were flooded with 4 ml of sterile deionized water containing 0.1% Tween 20 and conidia were liberated by agitation using a sterile glass rod (Turechek and Stevenson 1998). The resulting conidial suspension was centrifuged at 6500 RPM for 6 min, the supernatant decanted, and the conidial pellet resuspended in 2 ml of sterile deionized water containing 0.1% Tween 20 (Cancro 2000). This solution was filtered through two layers of sterile cheesecloth and the concentration of conidia adjusted to 1×10^5 conidia/ml using a hemocytometer (Turechek and Stevenson 1998). Sensitivity of individual isolates to fentin hydroxide

and tebuconazole was determined by modifying a bulk-spore assay that was originally designed for leaf samples and described by Seyran et al. (2010). Technical-grade fentin hydroxide (98.7% a.i.; Chem Service, Inc., West Chester, PA) and tebuconazole (97.5% a.i.; Bayer CropScience, Research Triangle Park, NC) were each dissolved in acetone to reach concentrations of 10,000 and 3,000 µg/ml, respectively. The final concentration of 10 µg/ml fentin hydroxide in medium was obtained by adding 1 ml of the fungicide solution to 1 liter of autoclaved 2% water agar (WA; 20g agar per liter of water) that had cooled in a water bath to 50°C. Similarly, 1 ml of the tebuconazole stock solution was added to 1 liter of autoclaved and cooled 1/4 strength PDA (qPDA; 9.75 g PDA and 11.25 g agar per liter of water) to reach a final concentration of 3 µg/ml. Non-fungicide-amended WA and qPDA control medium was prepared by adding 1 ml acetone to 1 liter of WA or qPDA that had been autoclaved and cooled.

Conidial suspensions for each isolate were transferred in aliquants of 50 µl and spread across three replicate dishes of fentin-hydroxide-amended and non-amended WA, as well as three replicate dishes of tebuconazole-amended and non-amended qPDA. The dishes were incubated in the dark at room temperature (23 to 25ºC) for 48 h for fentin hydroxide, or 72 h for tebuconazole sensitivity testing. Using a compound microscope at a magnification of 100×, conidial germination was determined by arbitrarily selecting 50 conidia per replicate dish and counting the number that had germ tubes of a length of at least four times the diameter of the conidium. Fentin hydroxide sensitivity was then quantified as percent relative germination (RGe), the proportion of germinated conidia on the fentin-hydroxide-amended medium divided by the proportion of germinated conidia on the non-amended medium, multiplied by 100 (Seyran et al. 2010). For tebuconazole

sensitivity, ten individual micro-colonies were selected per replicate plate and measured using a compound microscope (100×) as described by Seyran et al. (2010). All micro-colony growth measurements were corrected for the average length of a germinated conidium and germ tube by subtracting 15 µm from all micro-colony measurements (Standish et al. 2018). Micro-colony growth was expressed as relative growth (RGr), calculated as the corrected mean diameter of ten colonies on tebuconazole-amended medium divided by the corrected mean diameter of ten colonies on non-amended medium and multiplied by 100. The assays to determine sensitivity to both fentin hydroxide and tebuconazole were conducted twice.

Fitness assessments. For each isolate, the following fitness components were evaluated: conidial production, germination ability, micro-colony growth, sensitivity to osmotic stress, and sensitivity to oxidative stress. Experiments for each component were conducted three times, each with three replicates, and the isolates used were maintained on PDA+ throughout the course of the study. To assess conidial production in vitro, a fungal slurry, made for each isolate as described previously, was pipetted in aliquants of 0.6 ml and spread across petri dishes (15 × 60 mm diameter) containing OA+. Cultures were incubated, and conidia were collected using the protocols described above. The concentration of conidia was estimated using a hemocytometer and was expressed as the number of conidia produced per mm^2 of colony area. Conidial germination and micro-colony growth were measured by collecting conidia from actively sporulating cultures of each isolate on OA+ as described above. The concentration of conidia was determined and adjusted to a final concentration of 1×10^4 conidia/ml using a hemocytometer (Turechek and Stevenson 1998). Conidial suspensions for each isolate were transferred

onto three dishes of WA and three dishes of qPDA in aliquants of 50 µl that were then spread across the respective media. The plates were placed in the dark and kept at ambient temperature (23 to 25ºC). Conidial germination was assessed after 48 h as described above, and micro-colony growth was measured after 72 h, also as described above with the exception that the measurements were corrected by subtracting 2 µm to represent the average length of a conidium. Sensitivity to osmotic stress was determined by comparing micro-colony growth of isolates on PDA amended with 2, 4, or 6% NaCl (20, 40, or 60 g NaCl per liter of PDA, respectively). Similarly, sensitivity to oxidative stress was determined by comparing micro-colony growth of isolates on PDA supplemented with 0.1, 1.0, or 10 mM hydrogen peroxide (H_2O_2; 3% stabilized solution). Micro-colony diameters were measured as previously described and were corrected by subtracting the average length of a conidium (2 µm).

Stability of phenotypic insensitivity to fentin hydroxide and tebuconazole. To test whether insensitivity to fentin hydroxide and tebuconazole is stable, sensitivity to both fungicides was determined for a subset of isolates on WA and qPDA before and after five aseptic transfers (Table 6.1). Conidia were collected from actively sporulating cultures of each isolate on OA+ as described above and a hyphal tip from the edge of the culture was transferred onto qPDA, amended with the previously mentioned antibiotics. These qPDA dishes were incubated at 25ºC in the dark for 14 days before a new hyphal tip transfer was made. In total, five transfers were made before conidia were produced and collected again, as previously described. For the initial and final generations of each isolate, conidial germination and micro-colony growth were assessed on medium amended with three concentrations of fentin hydroxide (1.0, 3.0, and 10.0 µg/ml) or

tebuconazole (1.0, 3.0, and 10.0 µg/ml). Fungicide-amended and non-amended media were prepared as previously described with the exception that concentrations of 1,000 and 3,000 µg/ml fentin hydroxide (final concentrations of 1.0 and 3.0 µg/ml in medium), and 1,000 and 10,000 µg/ml tebuconazole (final concentrations of 1.0 and 10.0 µg/ml in medium) were also included. Conidial germination was expressed as RGe and micro-colony growth was expressed as RGr as described above. Three replicate plates were prepared for each isolate and the experiment was conducted twice.

Data analysis. Statistical analyses of fitness components and phenotypic stability were performed separately for fentin hydroxide and tebuconazole sensitivity datasets, and residuals for each fitness component and stability test were assessed for normality. A linear mixed-model regression analysis was conducted using PROC MIXED in SAS (v. 9.4, SAS Institute, Cary, NC) to determine the effects of sensitivity on the previously described fitness parameters. In these analyses, sensitivity values for fentin hydroxide (RGe) or tebuconazole (RGr) were continuous variables and treated as covariates and experiment was a class variable. A non-parametric, paired t-test (Wilcoxon signed-rank test) was performed using PROC UNIVARIATE in SAS to determine significant differences between experimental repeats. The same test was used to compare RGe or RGr values on the three concentrations of each fungicide before and after five transfers on non-amended media.

Results

Fungicide sensitivity. A total of 20 isolates was chosen to be included in this study; however, only 17 and 16 of those were included for fitness testing of fentin

hydroxide and tebuconazole insensitivities, respectively. The mean fentin hydroxide sensitivity values on 10 µg/ml ranged from 0.0 to 16.4% while the mean tebuconazole sensitivity values on 3 µg/ml ranged from 0.0 to 81.9% (Table 6.1).

Fitness testing. For fentin hydroxide isolates, the residuals of conidial production and micro-colony growth on medium amended with 1.0 mM H_2O_2 violated the assumptions of normality. Similarly, for the isolates tested with tebuconazole, the residuals for conidial production and micro-colony growth on both 2% NaCl amended- and 1.0 mM H_2O_2 amended-media were non-normal. In all cases, non-normal variables were ln-transformed prior to analysis. For both fungicides, poor conidial germination was observed on 4% NaCl (between 5.5 and 35.0%), 6% NaCl (between 4.7 and 20.0%), and 10 mM H_2O_2 (between 4.4 and 24.4%), thus, micro-colony growth data were not collected and the components were omitted from further analysis. The effects of fentin hydroxide RGe values on combined results for sporulation (as conidial production per mm^2) and micro-colony growth on qPDA, 2% NaCl, 0.1 mM H_2O_2, and 1.0 mM H_2O_2 were not significant. The effects of fentin hydroxide RGe values on percent germination were examined across three experiments. In the first experiment, the two least sensitive isolates (P16FH2 and P16T3; Table 6.1) failed to produce enough conidia for germination testing, thus, the results of experiment 1 were not included in the final analysis. However, the combined results of experiments 2 and 3 showed a significant ($P = 0.0014$) and negative trend; percent germination decreased with increasing RGe values (Table 6.2; Fig. 6.1). For combined results for tebuconazole insensitivity, the effects of RGr on sporulation, percent germination, or micro-colony growth on qPDA, 2% NaCl, and 0.1 mM H_2O_2 were not significant. However, the effects of tebuconazole RGr values

on micro-colony growth on 1.0 mM H_2O_2 were significant (P = 0.0023) (Table 6.2). The combined experiments revealed that ln-transformed micro-colony growth on 1.0 mM H_2O_2 decreased linearly with increasing RGr on tebuconazole-amended medium (Fig. 6.2).

Stability of phenotypic insensitivity to fentin hydroxide and tebuconazole. Experimental repeats for fentin hydroxide RGe and tebuconazole RGr values on all three concentrations before and after transfers did not differ significantly and were combined (data not shown). For fentin hydroxide, the mean RGe values on 1.0 µg/ml before and after transfer were 92.5 and 72.8%, respectively, and represented a significant decrease in RGe (Table 6.3). Similarly, the mean RGe values before and after transfer on 3.0 µg/ml were 72.2 and 35.5%, respectively, and the decrease was significant (Table 6.3). The mean RGe before and after transfers on 10 µg/ml did not differ significantly but did decrease from 7.5 to 3.2%. Mean tebuconazole RGr values on 1.0, 3.0, and 10.0 µg/ml before and after transfers were 56.2 and 55.3%, 31.0 and 19.8%, and 2.8 and 2.3%, respectively, and did not differ significantly before or after transfers on any of the three concentrations tested (Table 6.3).

Discussion

Understanding the fitness components of fentin-hydroxide- and tebuconazole-resistant and sensitive isolates of *V. effusa* could aid in the development of additional resistance management strategies to combat fungicide resistance in *V. effusa* (Brent and Hollomon 2007). Thus, the focus of this study was to assess fitness attributes of isolates of *V. effusa* with varying levels of insensitivity to fentin hydroxide or tebuconazole, and

to identify whether these insensitivities were stable after several successive vegetative transfers. These results provide evidence of fitness penalties associated with both fentin hydroxide and tebuconazole insensitivity. Conidia produced by fentin-hydroxide-insensitive isolates were less viable than those produced by more sensitive isolates and insensitivity was not stable; insensitivity decreased significantly after five consecutive transfers on non-amended media. Additionally, isolates with greater insensitivity to tebuconazole grew less than sensitive isolates on media amended with 1.0 mM H_2O_2. If the oxidative burst associated with pathogen recognition in other plants occurs in pecan (Torres et al. 2006), these isolates would be expected to grow less within the plant after infection. However, tebuconazole RGr values calculated before transfers did not differ significantly from those determined after transfers, indicating that tebuconazole insensitivity is phenotypically stable.

In the U.S., fentin hydroxide is only labelled for use on pecan, potato, and sugar beet [(as Super Tin 4L (United Phosphorus, Inc., King of Prussia, PA) and Agri Tin (Nufarm Americas, Inc., Burr Ridge, IL)]. Within this limited number of crops, practical resistance to fentin hydroxide has become an issue for only one other pathogen. In the case of the sugar beet leaf spot pathogen, *Cercospora beticola*, isolates tolerant to the organotin fungicides fentin acetate and fentin chloride were first described in 1976 (Giannopolitis 1978) and were subsequently observed to cause less severe leaf spot epidemics on untreated sugar beets when compared with more sensitive isolates (Giannopolitis and Chrysayi-Tokousbalides 1980). Isolates of *C. beticola* insensitive to fentin hydroxide were first reported in the U.S. in 1994, but interestingly, were found to decrease in frequency when annual applications of fentin hydroxide declined between

1998 and 2008 (Bugbee 1995; Secor et al. 2010). A similar phenomenon, although on a shorter timescale, was observed for *V. effusa* where RGe values, on 30 µg/ml fentin hydroxide, of isolates collected in September 2016 were over 10 times greater than those isolates collected from the same trees in June 2017 (Standish et al. 2018). The effect of fentin hydroxide sensitivity on the ability of these isolates to infect pecan is currently unknown. Attempts to inoculate several isolates were unsuccessful due to the variable length of leaflet susceptibility coupled with the time required to produce the required inoculum. Nevertheless, the results generated herein provide evidence that more insensitive isolates produce less viable conidia, which, in the absence of the fungicide, would lead to a reduction of insensitive isolates in the overall *V. effusa* population. The pathogen overwinters as stromata within lesions on the leaves, shoots, and shucks of pecan trees and conidia are borne on those stromata in the spring when new leaves are unfolding (Demaree 1924). If these stromata were initially produced by fentin hydroxide insensitive isolates, then it is possible that conidia produced from those stromata could be less viable and result in a lower frequency of insensitive isolates in the initial inoculum population at the beginning of a given year. Whether this occurs in commercial orchards is not known, and future research is needed to better understand the epidemiological consequences of any fitness penalties associated with fentin hydroxide insensitivity in isolates of *V. effusa*. After five consecutive transfers on a fungicide-free medium, fentin hydroxide RGe values decreased significantly when tested on 1.0 and 3.0 µg/ml, respectively. This provides evidence that corroborates the field-based observations of Secor et al. (2010) for *C. beticola* and Standish et al. (2018) for *V. effusa* where reductions in the overall frequency of resistant isolates, or RGe values, decreased

substantially in the absence of fentin hydroxide applications. Taken together, these results indicate that fentin hydroxide insensitivity may not be stable, which suggests that the mechanism of resistance is not a target site mutation, but a temporary adaptation of the pathogen to the fungicide. The mechanism of resistance by which pathogens overcome organotin activity is not well understood and merits further examination in *V. effusa*.

The DMIs are a large group of broad-spectrum fungicides that exhibit systemic properties, making them valuable for the control of a wide range of plant pathogens (Ziogas and Malandrakis 2015). Resistance to these fungicides typically follows a quantitative development pattern which has led to practical resistance in populations of certain pathogens, including *V. effusa* (Standish et al. 2018; Ziogas and Malandrakis 2015). Fitness costs have been associated with DMI insensitivity for some pathogens but not for others. When flutriafol-resistant and sensitive isolates of *C. beticola* were compared, resistant isolates were less virulent, and produced fewer conidia than their sensitive counterparts (Karaoglanidis et al. 2001). Furthermore, the frequency of flutriafol-resistant isolates decreased significantly in competition with sensitive isolates (Karaoglanidis et al. 2001). The results of a follow-up study provide evidence that flutriafol sensitivity was stable after consecutive transfers on non-amended media but that an increase in sensitivity occurred in isolates exposed to cold conditions (Karaoglanidis and Thanassoulopoulos 2002). A similar phenomenon was observed in isolates of *V. inaequalis*; fusilazole sensitivity was stable after 10 months of sub-culturing but sensitivity increased considerably for the same isolates after 7 months of storage at 2ºC (Köller et al. 1991). Interestingly, instability of propiconazole resistance was observed in

isolates of *Monilinia fructicola*; percent inhibition increased significantly following both consecutive transfers in vitro and months in cold storage (Cox et al. 2007). For those resistant isolates, conidial production, conidial germination, and growth rate were reduced but this was not a consistent observation across all evaluation periods (Cox et al. 2007). In the current study, tebuconazole sensitivity was phenotypically stable after five consecutive transfers and cold storage conditions were not considered for testing. However, micro-colony growth on 1.0 mM H_2O_2 was negatively correlated with tebuconazole insensitivity. Evidence of hypersensitivity to oxidative stress in fungicide-resistant isolates has been observed in succinate-dehydrogenase-inhibitor-resistant isolates of *Alternaria alternata* collected from pistachio and peach (Avenot et al. 2009; Fan et al. 2015). The concentration of hydrogen peroxide in plant tissues is in the micromole to low millimole range (Cheeseman 2006; Møller et al. 2007), which corresponds with the concentrations used in this study. As previously mentioned, attempts to inoculate pecan leaflets with isolates of *V. effusa* were unsuccessful, hence the predicted cost of oxidative stress associated with tebuconazole insensitivity could not be directly tested on the host plant. Nevertheless, this study provides evidence that tebuconazole-insensitive isolates are more likely to be sensitive to an oxidative burst, similar to that which often follows successful pathogen recognition in the plant compared to sensitive isolates (Torres et al. 2006).

In this study, we have provided evidence that fentin-hydroxide-insensitive isolates produce less viable conidia than do sensitive isolates. Likewise, tebuconazole-insensitive isolates were more sensitive to oxidative stress than were isolates sensitive to tebuconazole. Fentin hydroxide insensitivity to concentrations of 1.0 and 3.0 µg/ml was

not stable while insensitivity to three concentrations of tebuconazole proved to be stable after five mycelial transfers. Further research is required to investigate the epidemiological consequences of fitness penalties associated with resistance to these two fungicides. However, the evidence that fentin hydroxide resistance is not stable means that a resistant *V. effusa* population may shift towards sensitivity in the absence of selection to this important fungicide and therefore provides a crucial building block for resistance management strategies.

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Table 6.1. Year of collection and sensitivity to fentin hydroxide and tebuconazole of *Venturia effusa* isolates used in this study

		Fungicide sensitivity		Phenotypic stability[a]	
Isolate	Year	RGe[b]	RGr[c]	Fentin hydroxide	Tebuconazole
TC6	1997	5.8 (0.3)	0.0 (---)	×	×
TC17	1997	2.1 (1.0)	0.0 (---)		
P16AT1	2016	4.4 (1.2)	---	×	
P16AT2	2016	0.0 (---)	25.4 (3.0)	×	
P16AT7	2016	0.0 (---)	81.9 (4.4)		
P16FH1	2016	7.4 (5.2)	52.3 (13.7)		
P16FH2	2016	16.4 (3.3)	57.2 (1.8)	×	×
P16FH3	2016	0.0 (---)	---		
P16FHT1	2016	3.4 (0.4)	47.7 (15.4)	×	×
P16FHT2	2016	10.5 (3.0)	---		
P16NTC1	2016	4.1 (1.5)	54.8 (0.2)	×	×
P16NTC2	2016	8.4 (1.3)	---		
P16T1	2016	0.0 (---)	10.8 (7.4)		×
P16T2	2016	0.0 (---)	22.1 (2.0)	×	
P16T3	2016	13.3 (2.9)	43.0 (8.0)		
P16T4	2016	---	51.7 (3.0)		
P16T5	2016	0.0 (---)	13.5 (7.4)	×	×
P16T6	2016	0.0 (---)	71.3 (1.0)	×	×
P171G	2017	---	16.4 (0.7)		
P171T	2017	---	46.3 (5.7)		×

[a]Phenotypic stability of fentin hydroxide and tebuconazole insensitivity was assessed on subsets of *Venturia effusa* isolates indicated by "×".

[b]RGe = relative germination calculated as the proportion of germinated conidia on medium amended with 10.0 µg/ml fentin hydroxide divided by the proportion of those germinated on nonamended medium and multiplied by 100. Values are percentages with standard errors in parentheses.

[c]RGr = relative growth, calculated by taking the corrected mean diameter of ten micro-colonies on media amended with 3.0 µg/ml tebuconazole and multiplied by 100. Values are percentages with standard errors in parentheses.

Table 6.2. Significance levels (*P*-values) from linear mixed-model regression analyses to determine overall effects of initial sensitivity to fentin hydroxide and tebuconazole on fitness components in isolates of *Venturia effusa*[a]

				Osmotic and oxidative stress[b]		
Fixed effects[c]	**Sporulation (mm^2)**	**Germination (%)**	**Micro-colony growth (µm)**	**2% NaCl**	**0.1 mM H_2O_2**	**1.0 mM H_2O_2**
Fentin hydroxide						
Exp	0.7573	0.3476	0.8698	0.5672	0.9702	0.7210
RGe	0.2664	**0.0014**	0.3488	0.6121	0.2036	0.3748
RGe*Exp	0.3326	0.9983	0.5083	0.1625	0.6110	0.7477
Tebuconazole						
Exp	0.9085	0.5718	0.3121	0.5624	0.4980	0.2726
RGr	0.1324	0.3818	0.5767	0.7595	0.8847	**0.0023**
RGr*Exp	0.8767	0.8205	0.2029	0.6089	0.5034	0.6977

[a]Significant effects of experiment, initial sensitivity (RGe or RGr), and the combinations are bold-faced ($P \leq 0.05$). The *P*-values for sporulation and 1.0 mM H_2O_2 for both fentin hydroxide and tebuconazole, and the values of 2% NaCl for tebuconazole are based on an analysis of ln-transformed data.

[b]Osmotic and oxidative stress assessed as micro-colony growth (µm) on media amended with 2% NaCl or 0.1 and 1.0 mM H_2O_2, respectively; growth on 4 and 6% NaCl and 10 mM H_2O_2 is not shown.

[c]Abbreviations: Exp = experiment; RGe = relative germination on fentin-hydroxide-amended medium (10 µg/ml); RGr = relative growth on tebuconazole-amended medium (3 µg/ml).

Table 6.3. In vitro sensitivity of *Venturia effusa* isolates to fentin hydroxide and tebuconazole prior to and after five consecutive transfers on ¼ strength potato dextrose agar

	Fentin hydroxide[a]			Tebuconazole[b]		
Concentration	**Before**	**After**	***P* value[c]**	**Before**	**After**	***P* value[c]**
1.0 µg/ml	92.5 (1.8)	72.8 (4.3)	**0.0008**	56.2 (6.5)	55.3 (7.2)	0.6685
3.0 µg/ml	72.2 (5.6)	35.5 (4.6)	**0.0003**	31.0 (5.1)	19.8 (4.4)	0.1439
10.0 µg/ml	7.5 (2.1)	3.2 (1.0)	0.1706	2.8 (1.0)	2.3 (0.9)	0.6848

[a]Values are relative germination (RGe): the proportion of germinated conidia on medium amended with 1.0, 3.0, or 10.0 µg/ml fentin hydroxide divided by the proportion of germinated conidia on the nonamended medium and multiplied by 100. Values are the means of nine isolates with standard errors in parentheses taken before and after five hyphal-tip transfers on non-amended medium.

[b]Values are relative growth (RGr): the corrected mean diameter of ten micro-colonies on medium amended with 1.0, 3.0, or 10.0 µg/ml tebuconazole and multiplied by 100. Values are the means of eight isolates with standard errors in parentheses taken before and after five hyphal tip transfers on non-amended media.

[c]*P*-values based on the Wilcoxon signed-rank test, significant effects are bold-faced ($P \leq 0.05$).

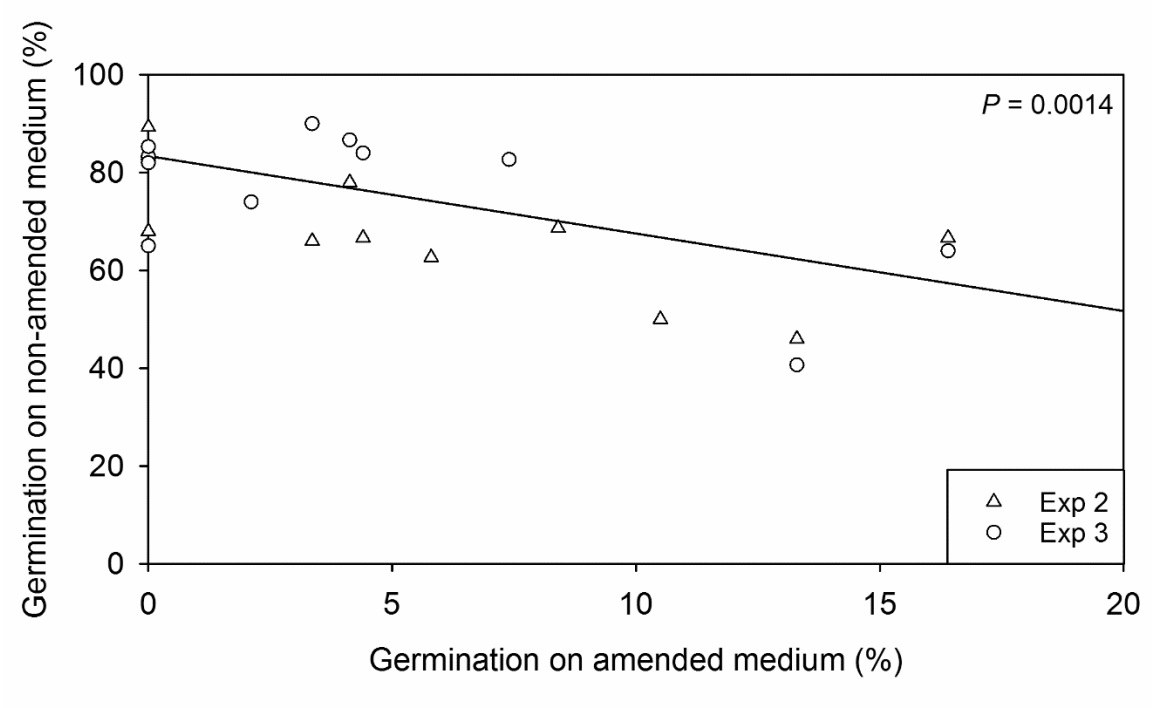

Figure 6.1. Relationship between sensitivity of *Venturia effusa* isolates to fentin hydroxide, measured as percent relative conidial germination on water agar amended with 10 µg/ml fentin hydroxide (x), and percent conidial germination on nonamended water agar (y). Data points are the results of two experimental repeats. The linear equation is given as y = 83.4 - 1.58x.

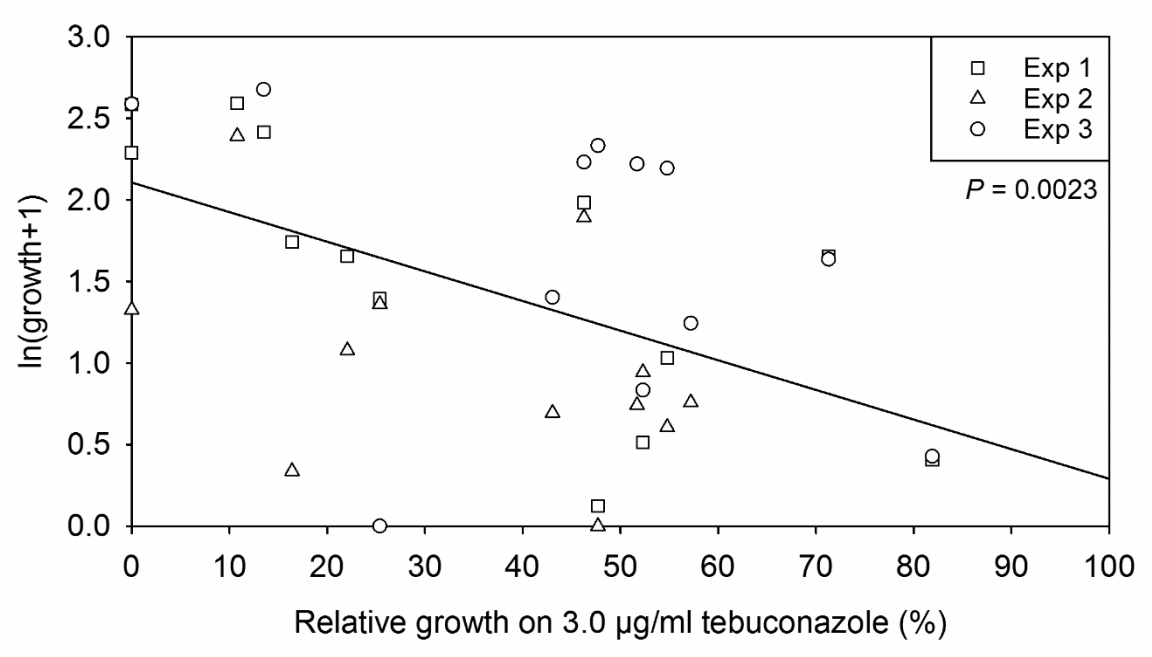

Figure 6.2. Relationship between sensitivity of *Venturia effusa* isolates to tebuconazole (x) and ln-transformed micro-colony diameter (µm) on medium amended with 1.0 mM H_2O_2 (y). Data points are the results of three experimental repeats. The linear equation is given as y = 2.11 – 0.018x.

CHAPTER 7

SUMMARY AND CONCLUSIONS

As the most yield-limiting disease afflicting pecan, scab is the primary target of disease management strategies in the humid Southeast (Littrell and Bertrand 1981). The use of resistant cultivars is a very effective method for scab management, but changing cultivars in an established orchard is not a simple endeavor. The scab pathogen, *Venturia effusa*, exhibits great genetic and pathogenic diversity allowing for its adaptation to cultivars with scab resistance (Bock et al. 2017; Conner and Stevenson 2004; Demaree and Cole 1929; Sparks 1992). Furthermore, mating type idiomorphs have been identified and the development of a sexual structure (a pseudothecium producing ascospores) has been observed in vitro (Bock et al. 2017; Charlton et al. 2016). Sexual reproduction occurring in the field may provide one avenue by which this pathogen might overcome host resistance (Bock et al. 2017). In practice, the most effective method for managing scab is to make multiple preventive fungicide applications throughout the growing season (Brock and Bertrand 2007). The high reproductive capacity of *V. effusa* leads to rapid population growth and an increased opportunity for the selection of fungicide-resistant individuals within the population (FRAC 2013). Isolates of *V. effusa* resistant to the methyl benzimidazole carbamate (MBC) active ingredient benomyl were first reported in 1975 after just 3 years of effective control (Littrell 1976). Additionally, a significant reduction in sensitivity to demethylation inhibitor (DMI) active ingredients was detected when comparing monoconidial isolates from 1995 with those collected in 2003

(Stevenson et al. 2004). In 2008, reduced sensitivities to dodine and triphenyltin hydroxide were documented in samples of *V. effusa* from Georgia when compared to baseline samples (Seyran et al. 2010).

To investigate the risk of QoI fungicide resistance in *V. effusa*, 125 isolates were collected from 16 counties in Georgia throughout the course of the 2014 fungicide sensitivity monitoring program described by Stevenson et al. (2015). Primers were designed to amplify a region within the cytochrome *b* gene associated with amino acid substitutions known to confer QoI resistance (F129L, G137R, and G143A). Sequence analysis of the 1,919-bp fragment revealed that a 1,407-bp intron was present immediately downstream of position 143 in all 125 isolates (Chapter 2). No sequences coding for amino acid substitutions were found at positions 129 or 143, but in seven isolates, a serine residue was present at position 137 in place of glycine (G137S). At the time, a reliable method for determining QoI sensitivity was not available due to the fungitoxic effects of alternative oxidase inhibitors on *V. effusa* in vitro. Thus, a detached leaf assay was developed to quantify the sensitivity of *V. effusa* isolates to azoxystrobin, and compare wild-type isolates with those carrying the G137S substitution identified in this work (Chapter 3). A total of 59 isolates, collected in 1997, 2014, 2016, and 2017, was characterized using this assay and included 45 wild-type and 14 carrying G137S. Based on the respective population medians, the G137S isolates were approximately eight times less sensitive than the wild-type isolates; however, there was considerable overlap between the two groups. Additionally, the levels of insensitivity associated with G137S were more similar to the partial resistance observed in isolates of *Pyrenophora tritici-repentis* exhibiting G137R or F129L (Sierotzki et al. 2007) than the levels of

complete resistance observed with G143A. In fact, due to the presence of the intron adjacent to position 143, the G143A substitution is not likely to occur in *V. effusa* indicating that the risk of complete QoI fungicide resistance is low. However, QoI resistance may still develop through intron loss events, the selection of genotypes that lack the intron, or as the result of other mutations in the gene (such as G137S). Moreover, the effects of G137S on QoI efficacy in Georgia pecan orchards are currently unknown and merit further exploration.

In vitro bioassays have been used to detect shifts in fungicide sensitivity over time, but a decrease in sensitivity in vitro is not necessarily associated with occurrence of practical resistance in the field. The relationship between in vitro sensitivity of *V. effusa* to fentin hydroxide or tebuconazole and efficacy of these fungicides under field conditions was explored over two growing seasons (Chapter 4). Scab epidemics on trees receiving one of six full-season fungicide programs (10 applications per season), including fentin hydroxide, tebuconazole, azoxystrobin, azoxystrobin plus tebuconazole, fentin hydroxide plus tebuconazole, or no fungicide (NTC) were compared. Maximum leaf scab incidence and area under the disease progress curve (AUDPC) in 2016 was significantly reduced by all fungicide treatments when compared with the nontreated control trees. However, Abound, Abound plus Orius 3.6F, and Super Tin 4L plus Orius 3.6F significantly reduced maximum leaf scab incidence and AUDPC when compared with the stand-alone Super Tin 4L and Orius 3.6F treatments. For 2017, maximum leaf scab incidence and AUDPC were significantly reduced on trees treated with Abound or Abound plus Orius 3.6F when compared with all other fungicide treatments. All fungicides significantly reduced AUDPC when compared with the nontreated and the

stand-alone Super Tin 4L or the tank mixture of Super Tin 4L plus Orius 3.6F treatments significantly reduced maximum leaf scab incidence when compared with the nontreated. The effects of fungicide treatment on maximum nut scab incidence were not significant in either 2016 or 2017 but maximum nut scab severity was reduced significantly in both years with treatments of Abound, Abound plus Orius 3.6F, Super Tin 4L, or Super Tin 4L plus Orius 3.6F when compared with the nontreated.

Samples of conidia were collected from individual trees and insensitivity to fentin hydroxide or tebuconazole was recorded as relative conidial germination (RGe) or micro-colony growth (RGr), respectively. Insensitivity to fentin hydroxide increased during the 2016 growing season (June to September), decreased between growing seasons (September 2016 to June 2017), and then decreased slightly during the 2017 growing season (June to September). Insensitivity to tebuconazole decreased significantly during the 2016 growing season (June to September), increased significantly between seasons (September 2016 to June 2017), and decreased significantly during the 2017 growing season (June to September). The fentin hydroxide sensitivity and efficacy results revealed that relative insensitivity values between 0.6 and 40.9% could occur without causing a likely control failure, although efficacy was reduced compared with other treatments. The tebuconazole results revealed that relative insensitivity values between 34.6 and 69.3% were likely to result in a control failure on trees treated only with tebuconazole. The QoI fungicide, azoxystrobin, was included in this study as a treatment to assess efficacy of the commercial product in a full-season treatment program over 2 years. Disease control remained effective after 10 consecutive applications in both seasons and provided practical evidence of a reduced risk of resistance predicted by the

presence of the intron in the cyt *b* gene, as described above. Unfortunately, a consistent quantitative relationship between fungicide efficacy and sensitivity was not observed in this study, due in part, to possible interplot interference. Further investigation utilizing a greater number of experimental units (trees or individual terminals) could allow for a relationship to be identified.

In 2014, a fungicide sensitivity monitoring program was conducted in Georgia allowing pecan growers to submit scab-infected leaf samples for fungicide sensitivity testing. As part of this program, multiple samples were collected from several orchards, either at the same time or at different times within the growing season. In some cases, the measured level of sensitivity varied significantly among samples from the same orchard block; insensitivity values for fentin hydroxide ranged from 17 to 53%, while thiophanate-methyl values ranged from 56 to 104%. Whether this was due to biological variation in the pathogen population or an artifact of the sampling protocol is unclear. Therefore, part of this work aimed at characterizing the spatial variation and temporal dynamics of fungicide sensitivity within an orchard block. An 18-ha block of approximately 576 trees in a commercial pecan orchard was divided into 64 contiguous quadrats arranged in an 8×8 grid with nine trees per quadrat to determine the spatial and temporal variation in fungicide sensitivities (Chapter 5). Scab-infected leaflets were collected from the trees in each quadrat and sensitivity to fentin hydroxide, propiconazole, and thiophanate-methyl was determined. This study was conducted for 3 years and sensitivity to each of the three fungicides differed significantly among years. Sensitivity to fentin hydroxide differed significantly in 2016 when compared with 2015 or 2017, propiconazole differed significantly in 2015 when compared with 2016 or 2017,

and thiophanate-methyl differed significantly in all 3 years. Significant spatial autocorrelation was observed among quadrats in sensitivity to propiconazole in 2017 and to thiophanate-methyl in 2015 and 2017, which suggests aggregation, but not in any of the other fungicide by year combinations. Similarly, omnidirectional spatial dependency was observed for both propiconazole and thiophanate-methyl insensitivity in 2017, but not in any other fungicide by year combination. In the latter two cases, the range of spatial dependence was greater than 276.5 m and could not be estimated accurately due to the limited size of the sampling area. An additional sampling was conducted in all 3 years to identify an appropriate sampling size and pattern for fungicide sensitivity screening using the methods of Seyran et al. (2010). Results showed that regardless of the sampling pattern used, three groups of 15 leaflets is likely sufficient for propiconazole and thiophanate-methyl sensitivity testing; however, additional groups would improve the precision of the assay. Overall, the results of this study show biological variation in the *V. effusa* orchard population that differs in space and among growing seasons.

If fungicide-resistant individuals exist within a pathogen population and exhibit reduced fitness, that portion of the population will likely decline in the absence of selection pressure. In such cases, discontinuing use of the fungicide for a period of time could potentially restore efficacy of the fungicide in question when it is reintroduced. The results of objective 2 presented above showed that fentin hydroxide insensitivity decreased significantly between growing seasons as the pathogen overwintered (Chapter 4). This decrease was in the absence of fungicide exposure which, while only measured over one overwintering period, suggests that insensitivity to fentin hydroxide is not stable and incurs a fitness cost to this pathogen. Thus, for *V. effusa* isolates with a range of

sensitivities to fentin hydroxide and tebuconazole, the predicted fitness components of conidial production, conidial germination, micro-colony growth, sensitivity to osmotic stress, and sensitivity to oxidative stress were evaluated (Chapter 6). For fentin hydroxide, conidial germination percentage was negatively correlated with greater relative germination values on fentin-hydroxide-amended medium (10 µg/ml), while tebuconazole insensitivity (measured on medium containing 1 µg/ml) was negatively correlated with hypersensitivity to 1.0 mM H_2O_2. Phenotypic stability of resistance was assessed on concentrations of 1.0, 3.0, and 10.0 µg/ml of both chemicals prior to, and after five transfers on quarter-strength potato dextrose agar. Insensitivity to tebuconazole did not differ significantly after transfers for any of the three concentrations tested, but insensitivity to 1.0 and 3.0 µg/ml fentin hydroxide decreased significantly after five transfers. These results help to explain the decrease in insensitivity to fentin hydroxide between growing seasons observed in the previous study (Chapter 4) and suggest that fentin hydroxide insensitivity comes at the cost of conidial viability. As such, this insensitivity is likely to be the result of a temporary adaptation of the pathogen to the fungicide. Tebuconazole insensitivity, however, is phenotypically stable but may be moderated because more insensitive isolates appear to be hypersensitive to oxidative stress.

Overall, the results presented in this dissertation provide information on fungicide resistance in *V. effusa* and how it may be managed in the future. Complete QoI resistance was found to be unlikely to occur due to the intron present downstream of position 143 and results of Chapter 3 showed that the G137S substitution is a likely mechanism of partial resistance; however, although a complete control failure is unlikely, the impact of

this substitution on QoI efficacy in Georgia pecan orchards remains to be determined. The relationship between fungicide efficacy in the field and sensitivity in vitro was determined for both fentin hydroxide and tebuconazole, and while a consistent quantitative relationship was not observed, the results revealed that tebuconazole is likely to fail in the field when relative growth values are between 34.6 and 69.3%. Fungicide sensitivity values were found to vary spatially across a large commercial orchard over 3 years and the assay currently used for sensitivity testing is likely to be effective with three groups of 15 leaflets, although the results did fluctuate on a yearly basis depending on the fungicide tested. The results presented in Chapter 5 underscore the importance of collecting a sample that represents the orchard where fungicide sensitivity testing will be conducted and not just sampling from "hotspot" areas. Finally, the results presented in Chapter 6 revealed that fentin hydroxide insensitivity was not stable after consecutive transfers on a non-amended medium. This coupled with fentin hydroxide insensitivity being negatively correlated with lower conidial viability implies that fentin hydroxide insensitivity may not be a stable trait and that resistant *V. effusa* populations may regain sensitivity in the absence of fentin hydroxide exposure. Future research directions that should be considered are to:

1) Investigate the occurrence of isolates of *V. effusa* carrying the G137S substitution throughout Georgia pecan orchards and determine direct effects of this substitution on QoI fungicide efficacy;
2) Characterize the relationship between fungicide efficacy and in vitro sensitivity using single-terminal spray trials;

3) Determine the mechanisms of resistance to the demethylation inhibitor fungicides and/or fentin hydroxide;
4) Explore the vertical distribution of fungicide-resistant *V. effusa* in mature pecan trees;
5) Assess the epidemiological consequences of the predicted fitness penalties observed in Chapter 6 of this work.

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APPENDIX A

APPENDIX TO CHAPTER 4

Table A.1. Monthly rainfall totals (mm) and number of rain events during the growing seasons of 2016 and 2017 recorded at the University of Georgia Ponder Farm in Ty Ty, GA

	Total rainfall (mm)[z]		Total rain events[y]	
Month	**2016**	**2017**	**2016**	**2017**
March	156.72	41.15	8	3
April	139.70	79.25	6	3
May	65.79	78.74	4	5
June	122.17	249.17	4	15
July	39.12	46.48	4	7
August	94.49	83.31	9	6
September	148.08	74.93	5	2
Total	766.06	653.03	40	41

[a]Rainfall data between Mar 1 and Sep 30 were collected by an on-site weather station and obtained from the Georgia Automated Environmental Monitoring Network (www.georgiaweather.net).

[b]Total rain events were considered as days where 2.54 mm of rain occurred

Table A.2 Effects of bi-weekly fungicide applications on pecan scab epidemics affecting cv. Desirable[w]

		Leaf scab (%)				Nut scab (%)			
Year	**Treatment**	**Maximum incidence**		**AUDPC[x]**		**Maximum incidence**		**Maximum severity**	
2016[y]									
	Super Tin 4L 0.877 L/ha	14.8	ab	5.4	bc	74.5	b	6.8	cd
	Orius 3.6F 0.585 L/ha	23.4	a	8.5	ab	95.9	a	19.0	b
	Abound 8.77 L/ha	12.5	bc	3.3	cd	72.5	b	6.8	cd
	Abound 0.877 L/ha + Orius 3.6F 0.585 L/ha	8.9	c	2.3	d	64.2	b	3.6	d
	Super Tin 4L 0.877 L/ha + Orius 3.6F 0.585 L/ha	12.8	bc	4.1	c	81.7	ab	12.5	bc
	Nontreated	23.3	a	9.6	a	100.0	a	44.9	a
2017[z]									
	Super Tin 4L 0.877 L/ha	7.5	a	1.7	ab	81.9	ab	6.1	b
	Orius 3.6F 0.585 L/ha	5.1	a	1.6	ab	100.0	a	20.2	a
	Abound 8.77 L/ha	4.0	ab	0.9	bc	80.1	ab	2.6	b
	Abound 0.877 L/ha + Orius 3.6F 0.585 L/ha	2.2	b	0.5	c	65.2	b	2.4	b
	Super Tin 4L 0.877 L/ha + Orius 3.6F 0.585 L/ha	4.4	ab	1.2	ab	71.9	b	4.6	b
	Nontreated	6.2	a	2.4	a	100.0	a	40.0	a

[w]By year, means within columns followed by the same letter are not significantly different according to pairwise *t* tests of least squares means (α=0.05) based on the lognormal distribution.

[x]Area under the disease progress curve (AUDPC) values calculated based on assessments of leaf scab incidence from 25 May to 20 July 2016 and 31 May to 28 July 2017.

[c]Applications made in 2016: 18, 28 Apr; 12, 26 May; 9, 23 Jun; 7, 21 Jul; 4, 18 Aug.

[z]Applications made in 2017: 8, 21 Apr; 5, 18 May; 1, 16, 27 Jun; 13, 27 Jul; 10 Aug.

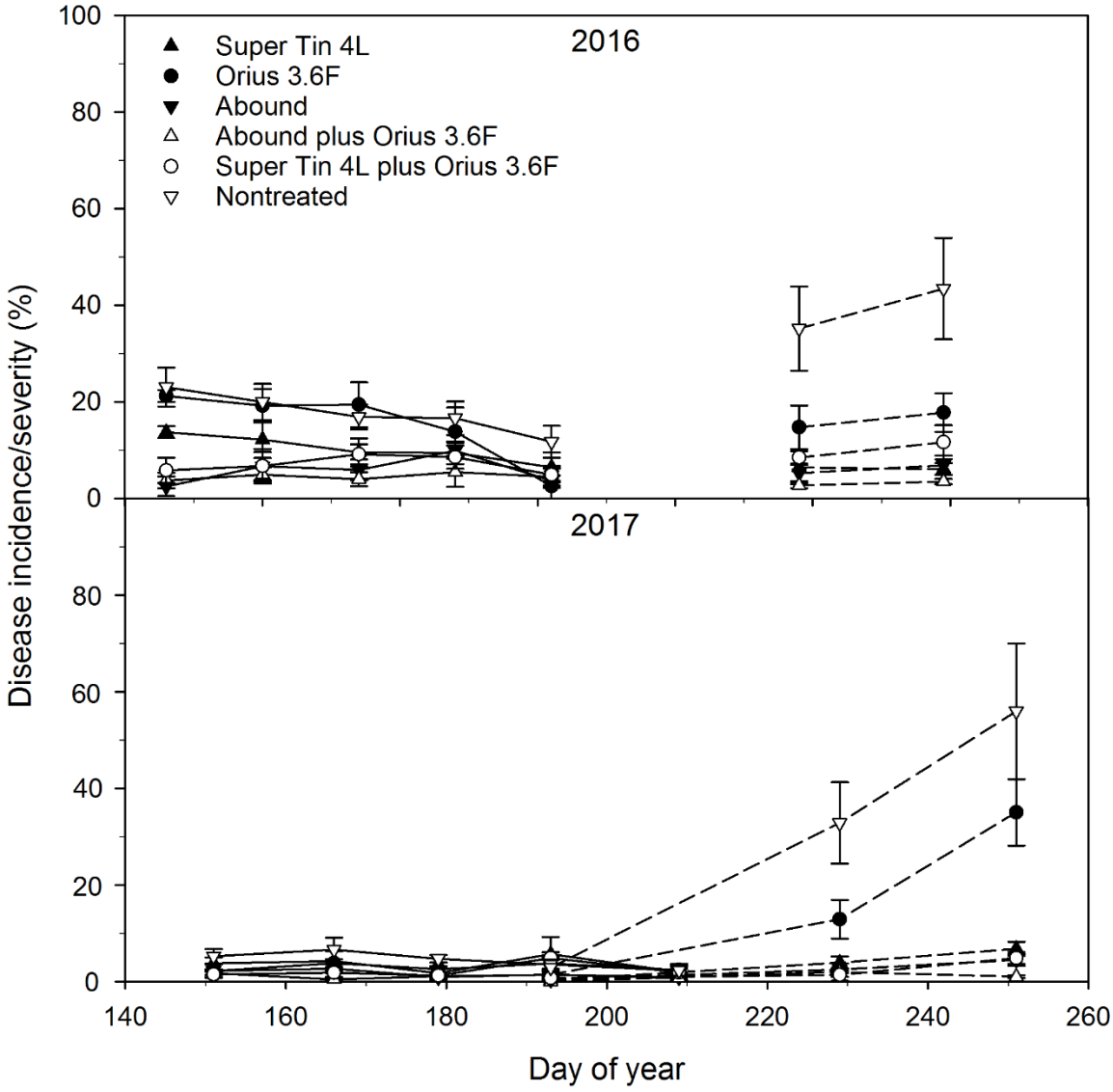

Figure A.1. Effect of bi-weekly fungicide applications on disease progress of pecan scab epidemics in field experiments on cv. Desirable in 2016 and 2017. Solid lines between symbols indicate incidence data collected on leaves while dashed lines between symbols indicate severity data collected from nuts.

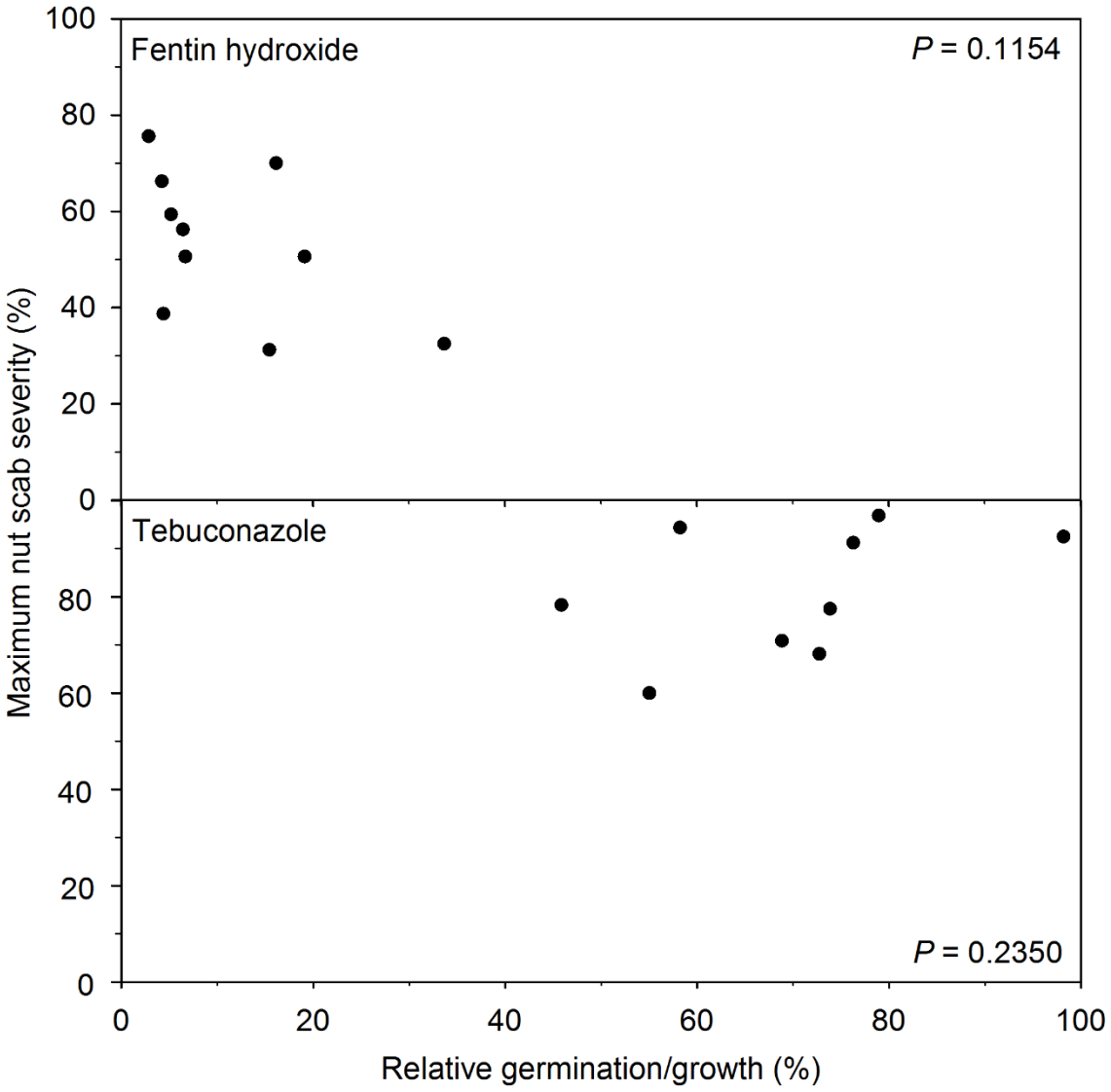

Figure A.2. Relationship between mid-season fungicide sensitivity to fentin hydroxide or tebuconazole and late-season nut scab severity. Fentin hydroxide sensitivity was assessed as relative germination on a discriminatory concentration of 30 µg/ml while tebuconazole sensitivity was assessed as relative growth on a discriminatory concentration of 1 µg/ml. All data were collected from cv. Wichita in 2016 and 2017.

APPENDIX B

APPENDIX TO CHAPTER 5

Table B.1. Season long fungicide programs used in the commercial pecan orchard in this study from 2015 to 2017

Year	Trade name	Active ingredient
2015	Super Tin + Topsin	TPTH[b] + thiophanate-methyl
	Super Tin + Elast	TPTH + dodine
	Super Tin + Topsin	TPTH + thiophanate-methyl
	Super Tin + Elast	TPTH + dodine
	Quadris Top	azoxystrobin + difenoconazole
	Super Tin	TPTH
	Monsoon + Sovran	tebuconazole + kresoxim-methyl
	Super Tin + Elast	TPTH + dodine
	Super Tin + Topsin	TPTH + thiophanate-methyl
2016	---[a]	---
2017	Agri Tin	TPTH
	Agri Tin	TPTH
	Agri Tin	TPTH
	Absolute + Tebuzole + Reliant	tebuconazole + trifloxystrobin, + tebuconazole + phosphorus acid
	Agri Tin + Kocide 3000 + Reliant	TPTH + copper hydroxide + phosphorous Acid
	Agri Tin + Reliant	TPTH + phosphorus acid
	Agri Tin + Reliant	TPTH + phosphorus acid
	Agri Tin + Topsin	TPTH + thiophanate-methyl
	Agri Tin	TPTH
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Elast + Reliant	TPTH + dodine + phosphorus acid
	Agri Tin + Reliant	TPTH + phosphorus acid

[a]Spray records from 2016 were unavailable
[b]TPTH = fentin hydroxide

Table B.2. Changes in the relative insensitivity of *Venturia effusa* to fentin hydroxide, propiconazole, and thiophanate-methyl from 2015 to 2017

		Change in RGe or RGr[a]			Signed rank[b]	
Fungicide	**Year**	**Mean**	**Median**	**Std Dev**	**S**	***P* value**
Fentin hydroxide	2015 to 2017	0.3	0.0	18.7	-26.5	0.8010
	2015 to 2016	6.0	7.6	15.8	620	<0.0001
	2016 to 2017	-5.7	-9.9	15.7	-482	0.0009
Propiconazole	2015 to 2017	36.3	45.2	38.8	796	<0.0001
	2015 to 2016	31.8	37.0	36.5	803	<0.0001
	2016 to 2017	4.6	1.2	30.4	157	0.2974
Thiophanate-methyl	2015 to 2017	-8.4	-6.9	40.9	-237	0.1136
	2015 to 2016	12.1	13.6	34.4	400	0.0065
	2016 to 2017	-20.5	-26.5	39.2	-604	<0.0001

[a]Change in percent relative growth or germination for samples from individual quadrats from 2015 to 2016, 2016 to 2017, and 2015 to 2017.
[b]Wilcoxon signed rank test indicating a significant change from 2015 to 2016, 2016 to 2017, and 2015 to 2017 (α=0.05).

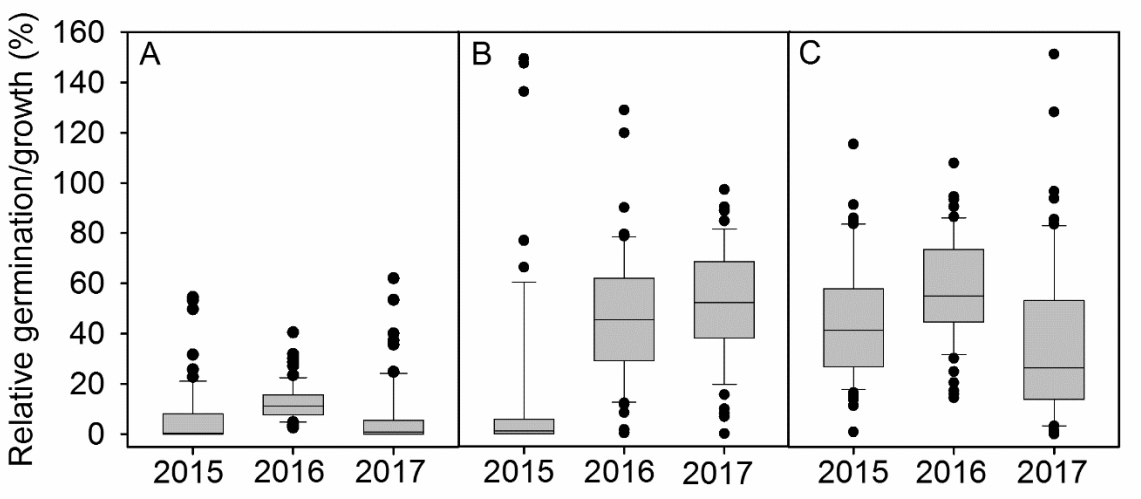

Figure B.1. Relative insensitivity of *Venturia effusa* samples collected across the same orchard from 2015 to 2017 (*n* = 64) to **A.** fentin hydroxide (30.0 µg/ml), **B.** propiconazole (1.0 µg/ml), and **C.** thiophanate-methyl (5.0 µg/ml).

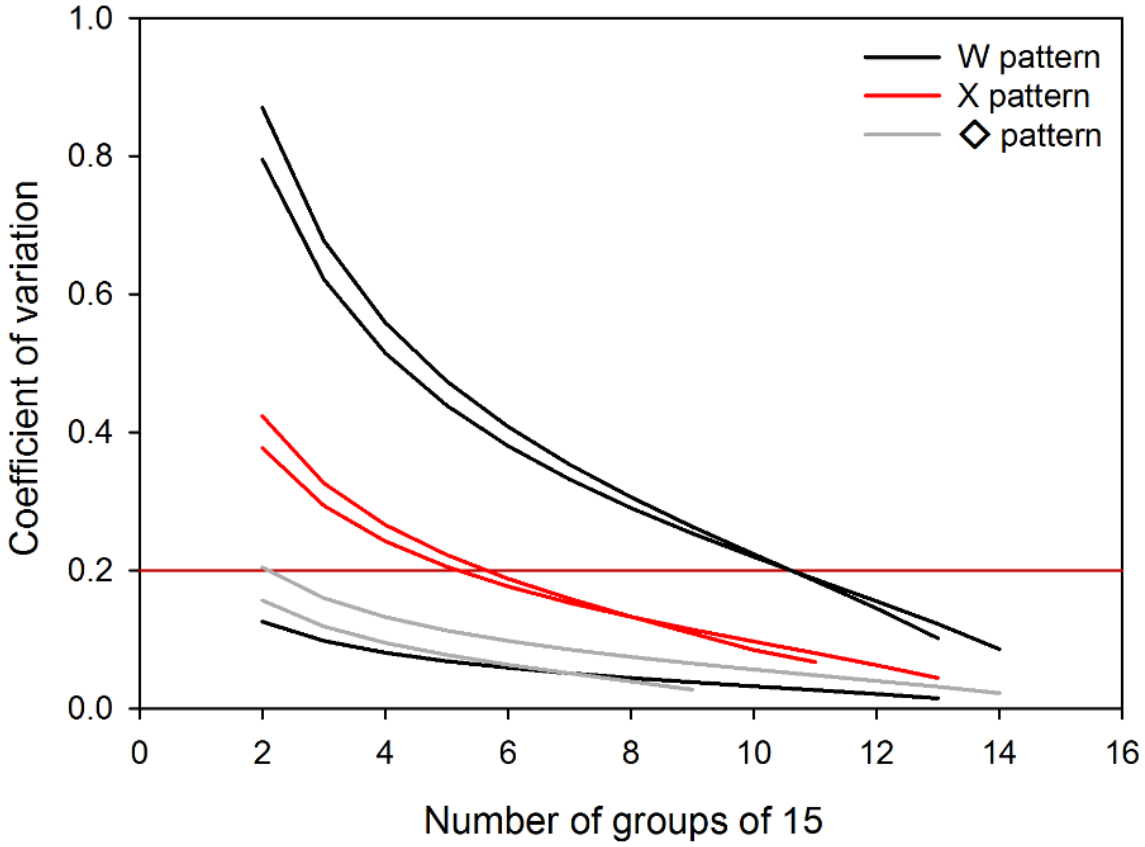

Figure B.2. Effect of sample size (as number of groups of 15 leaflets) and pattern on reliability of sensitivity testing for *Venturia effusa* on medium amended with propiconazole (1.0 µg/ml). Coefficient of variation expressed as (standard deviation for all possible combinations per sample size)/(mean of RGe or RGr values for those combinations). The horizontal line indicates a coefficient of variation of 0.2.

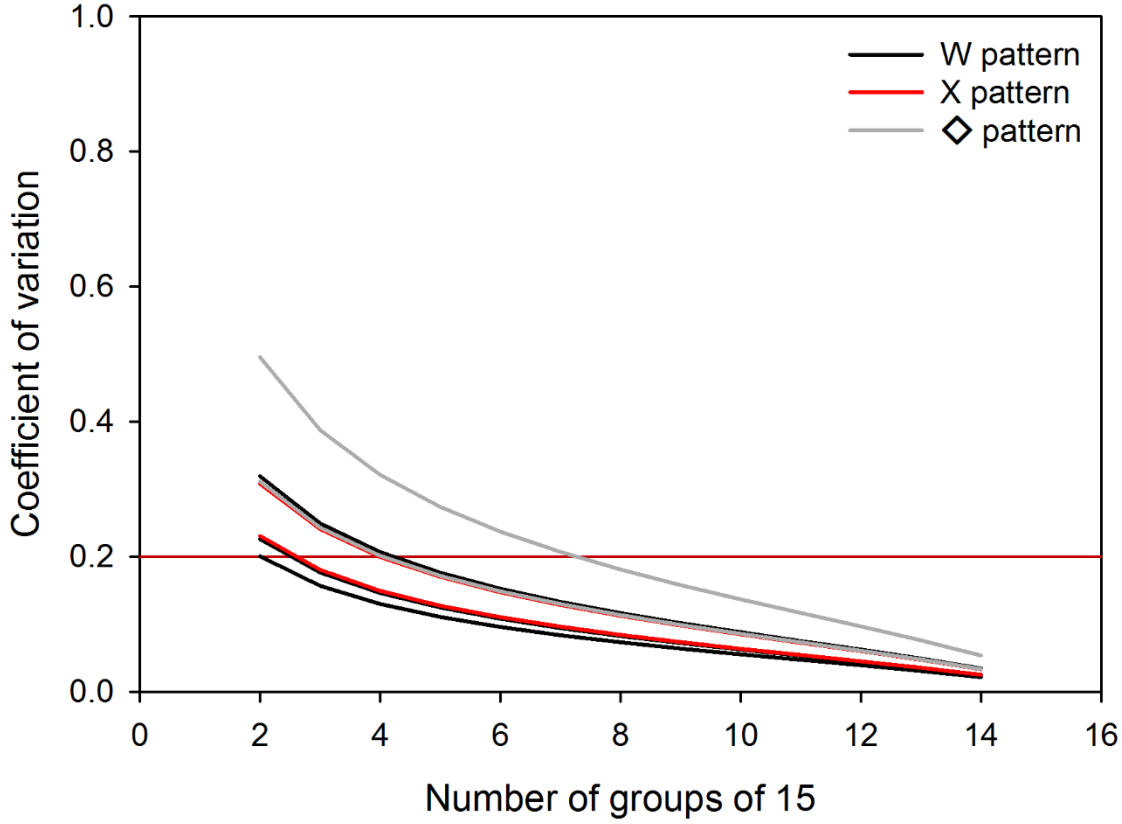

Figure B.3. Effect of sample size (as number of groups of 15 leaflets) and pattern on reliability of sensitivity testing for *Venturia effusa* on medium amended with thiophanate-methyl (5.0 µg/ml). Coefficient of variation expressed as (standard deviation for all possible combinations per sample size)/(mean of RGe or RGr values for those combinations). The horizontal line indicates a coefficient of variation of 0.2.

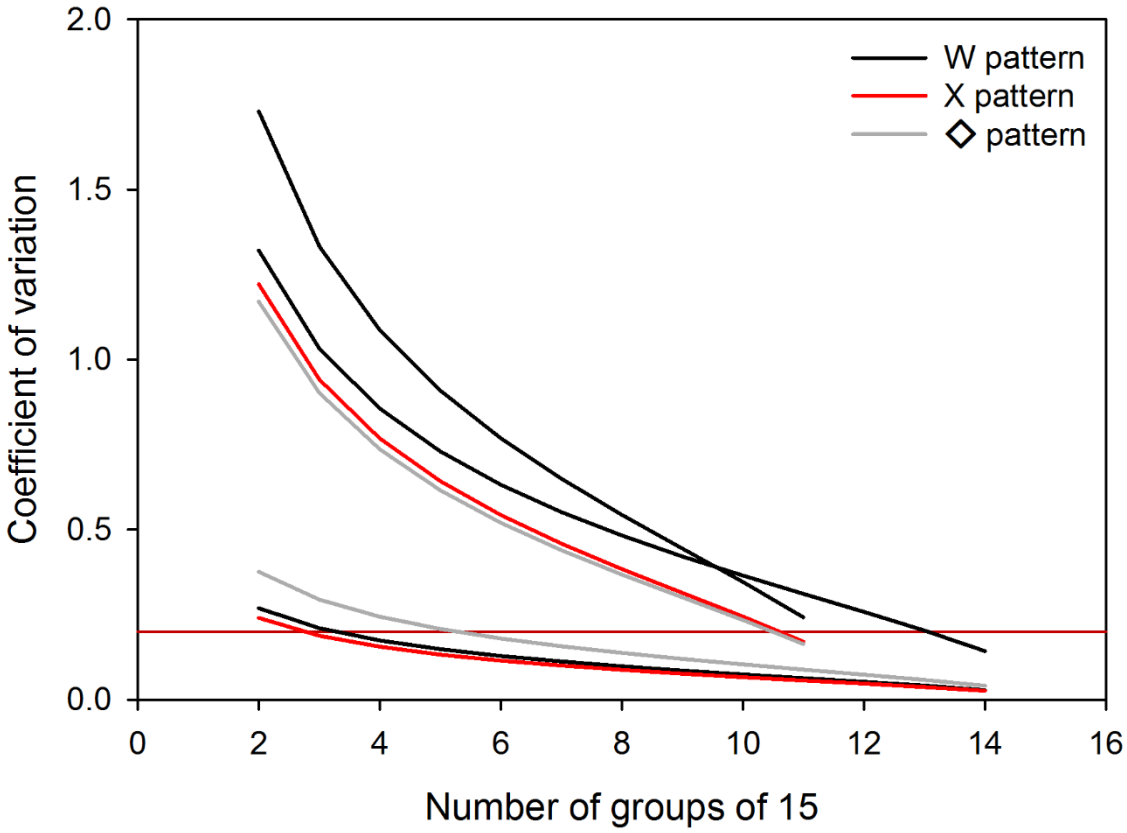

Figure B.4. Effect of sample size (as number of groups of 15 leaflets) and pattern on reliability of sensitivity testing for *Venturia effusa* on medium amended with fentin hydroxide (30.0 µg/ml). Coefficient of variation expressed as (standard deviation for all possible combinations per sample size)/(mean of RGe or RGr values for those combinations). The horizontal line indicates a coefficient of variation of 0.2.

APPENDIX C

APPENDIX TO CHAPTER 6

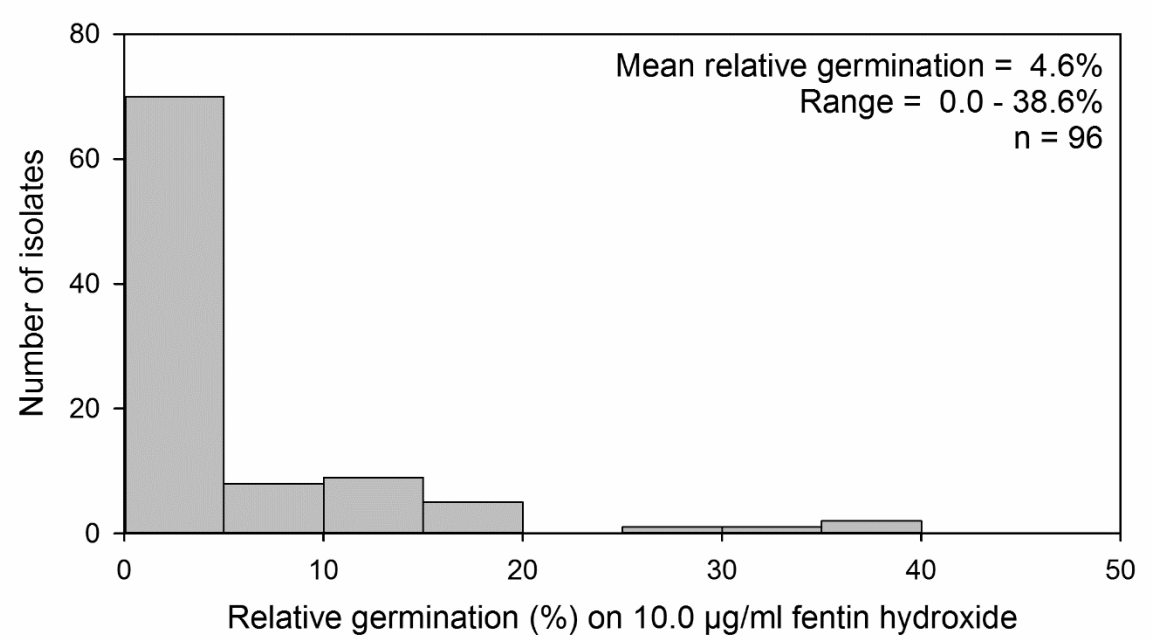

Figure C.1. Frequency distribution of percent relative germination values for *Venturia effusa* isolates tested on medium amended with 10.0 µg/ml fentin hydroxide to identify isolates for use in assessing fitness components and phenotypic stability of insensitivity.

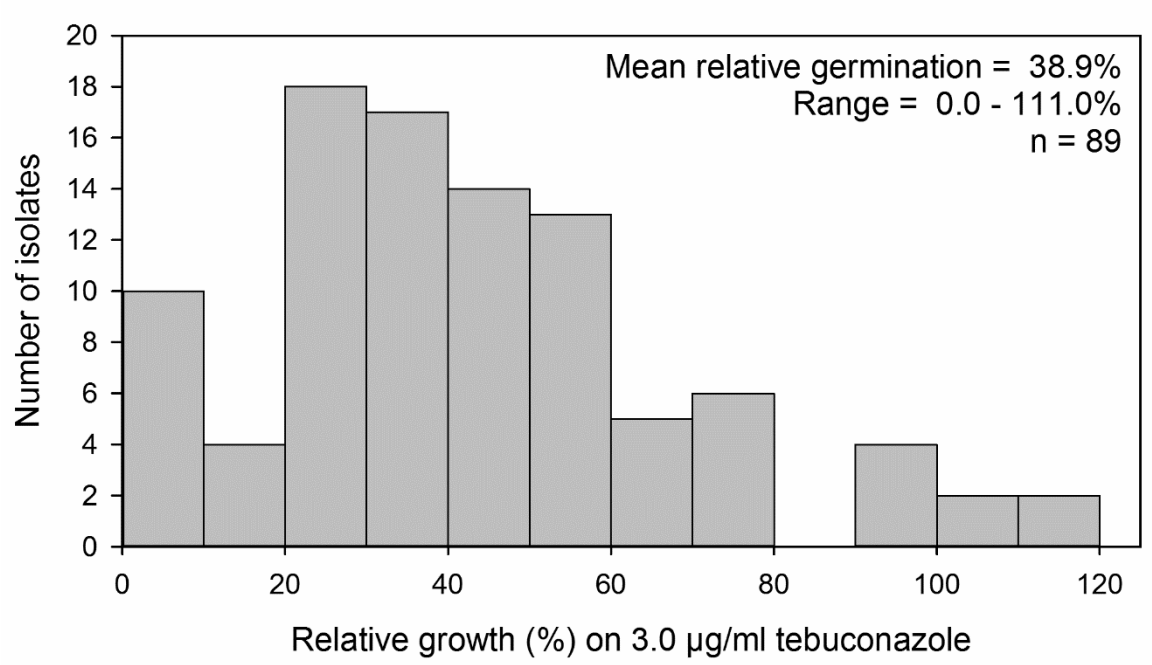

Figure C.2. Frequency distribution of percent relative growth values for *Venturia effusa* isolates tested on 3.0 µg/ml tebuconazole to identify isolates for use in assessing fitness components and phenotypic stability of insensitivity.

APPENDIX D

COMPARING AN IN VITRO BIOASSAY AND A MICROTITER PLATE TECHNIQUE TO DETERMINE SENSITIVITY OF *VENTURIA EFFUSA* TO FENTIN HYDROXIDE AND TEBUCONAZOLE

Rationale

Screening isolates of *Venturia effusa* using a traditional in vitro mycelial growth assay yields very consistent results but takes 6 weeks and requires that contamination constantly be checked for and removed. Seyran (2008) adopted a microtiter plate assay utilizing a 96-well plate and a plate reader to measure light penetrating through a slurry of mycelial fragments as a replacement for traditional mycelial growth assays. The proposed assay takes 7 days from start to finish and requires considerably less time and effort but needed validation to confirm that the results would relate to those generated using the traditional assays. Here, two separate sensitivity tests were conducted simultaneously using both assays for fentin hydroxide (18 isolates) and tebuconazole (28 isolates) to validate the microtiter plate assay as a fast and accurate replacement for the traditional method.

Isolate Preparation and Assay Methods

Isolates were cultured on potato dextrose agar (PDA; 39g per liter of water) and incubated for 6 weeks to generate sufficient biomass for sensitivity testing. Two 5-mm diameter plugs were removed from each fungal colony, including medium, and were placed into a sterile 2-ml microcentrifuge tube containing 1 ml of potato dextrose broth (PDB) and three sterile 3-mm glass beads. The fungal colonies were then macerated using a bead beating apparatus (Mini-Bead Beater, Biospec, Bartlesville, OK) for 1 min, before being transferred into a glass culture tube (16 × 100 mm) containing 5 ml of PDB. The slurry was mixed well using a vortex mixer and used in the procedures developed by Reynolds et al. (1997) and Seyran (2008), described below.

Petri dish-based method. Technical-grade fentin hydroxide (98.7% a.i.; Chem Service, Inc., West Chester, PA) and tebuconazole (97.5% a.i.; Bayer CropScience, Research Triangle Park, NC) were each dissolved in acetone to reach concentrations of 30,000 and 3,000 µg/ml, respectively. A final concentration of 30.0 µg/ml fentin hydroxide in medium was obtained by adding 1 ml of the fungicide solution to 1 liter of autoclaved 2% water agar (WA; 20g agar per liter of water) that had cooled in a water bath to 50°C. Similarly, a final concentration of 3.0 µg/ml was obtained by adding 1 ml of the tebuconazole stock solution to 1 liter of autoclaved and cooled 1/4 strength PDA (qPDA; 9.75 g PDA and 11.25 g agar per liter of water). Non-fungicide amended WA and qPDA control medium was prepared by adding 1 ml acetone to 1 liter of WA or qPDA that had been autoclaved and cooled. All media were poured into sterile petri dishes and once solidified, a sterile cork borer was used to remove a 5-mm diameter plug of agar to form a well in the center of each plate (Reynolds et al. 1997). The previously described fungal slurry was added into the 5-mm wells of each dish in aliquants of 50 µl, the dishes were wrapped in Parafilm, and kept in the dark at ambient temperature (23 to 25°C) for 6 weeks (Reynolds et al. 1997). After incubation, the diameter of each fungal colony was measured and adjusted by subtracting the diameter of the well (5 mm). The mean adjusted colony diameter was determined for each isolate and fungicide. Relative growth for each isolate was quantified as mycelial growth on the fungicide-amended medium divided by mycelial growth on the non-amended medium, multiplied by 100. This assay was conducted twice to determine both fentin hydroxide and tebuconazole sensitivity and included three replicate dishes per isolate and fungicide combination.

Microtiter plate-based method. Technical-grade fentin hydroxide and tebuconazole were dissolved as described above and added to potato dextrose broth to obtain the same final concentrations. For both fungicides, the fungicide-amended and non-amended broth were transferred in aliquants of 150 µl to each well of 96-well microtiter plates. The fungal slurries described above were then added in aliquants of 50 µl to each well of the microtiter plates. Initial light absorbance at a wavelength of 405 nm was determined for all microtiter plates using a Multiskan Plus Microplate Reader (Thermo Fisher Scientific, Waltham, MA) and Ascent Software (v. 2.6, Thermo Fisher Scientific, Waltham, MA). The plates were then wrapped in Parafilm and incubated in the dark at ambient temperature (23 to 25°C) for 7 days on a rotary shaker set at 100 rpm, at which point light absorbance was measured again (Seyran 2008). Relative growth for each isolate was quantified using the formula: relative growth = $(F_7 – F_0)/(C_7 – C_0)$, where F_0 and F_7 are the initial light absorbance values for wells with fungicide-amended medium and those taken on day 7, respectively; and C_0 and C_7 are the initial light absorbance values for wells with non-amended medium and those taken on day 7, respectively (Seyran 2008). Similar to the petri dish assay, the microtiter assay was conducted twice for both fungicides and included three replicate plates and three replicate wells within plates per isolate and fungicide combination.

Results and Discussion

The average relative growth values on fentin hydroxide for the plate and microtiter tests, were 17.8 and 11.6%, and 31.3 and 12.9%, in the first and second experiments respectively. For tebuconazole, the average relative growth values for the

plate and microtiter tests were 66.7 and 64.7%, and 64.5 and 62.1%, in the first and second experiments respectively. A regression analysis was performed separately for each experiment and fungicide with relative growth from the microtiter plate assay as the dependent variable and relative growth from the traditional assay as the independent variable. There was no significant linear relationship between the two methods for fentin hydroxide relative growth values measured on 30 μg/ml in either experiment (Fig. D.1). Similarly, in both experiments a significant linear relationship was not observed between the two methods for tebuconazole relative growth values measured on 3 μg/ml (Fig. D.2). This could potentially be due to the indirect measure of fungal growth (the light measurement taken using the microtiter plate assay) being compared with a direct measure of fungal growth (the in vitro bioassay), measurement errors as a result of possible clustering of mycelial fragments in the 96-well plates, or an equipment malfunction. Based on the results presented here, the microtiter plate method proposed by Seyran (2008) does not appear to be a suitable replacement for the traditional petri dish assay.

Literature Cited

Reynolds, K. L., Brenneman, T. B., and Bertrand, P. F. 1997. Sensitivity of *Cladosporium caryigenum* to propiconazole and fenbuconazole. Plant Dis. 81:163-166.

Seyran, M. 2008. Development of rapid in vitro assays and current status of fungicide sensitivity in the pecan scab pathogen *Fusicladium effusum*. M.Sc. thesis. University of Georgia, Athens.

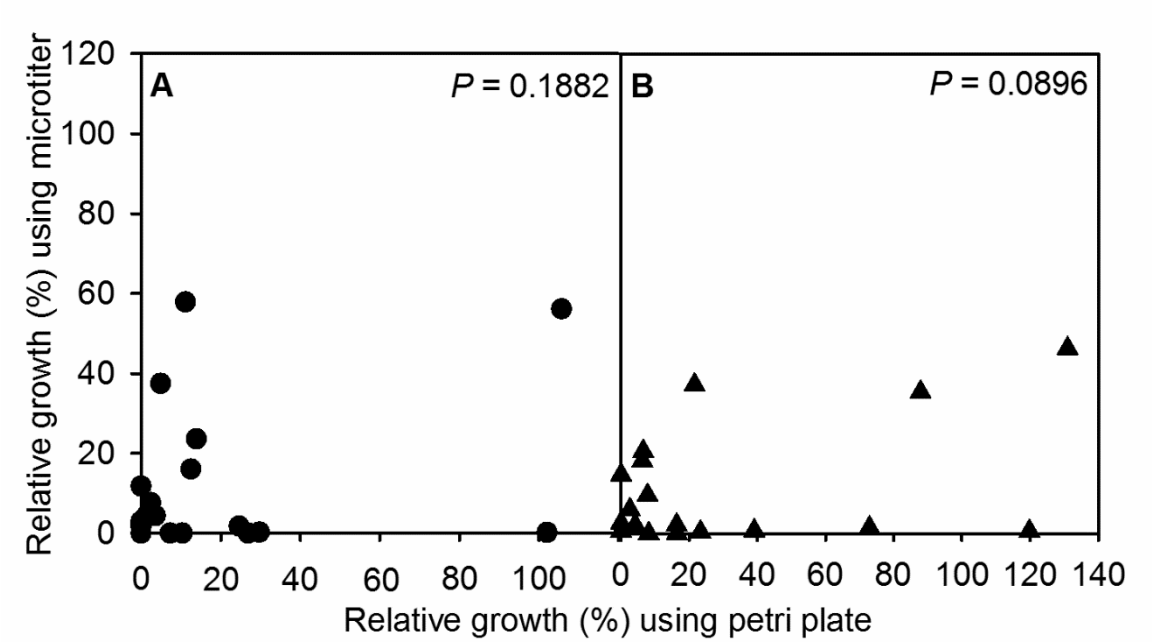

Figure D.1. Relationship between relative growth of *Venturia effusa* isolates to 30 µg/ml fentin hydroxide determined using a microtiter plate-based method and the traditional petri dish-based method. Values are the mean of replicates for each isolate. **A.** Experiment 1, **B.** Experiment 2.

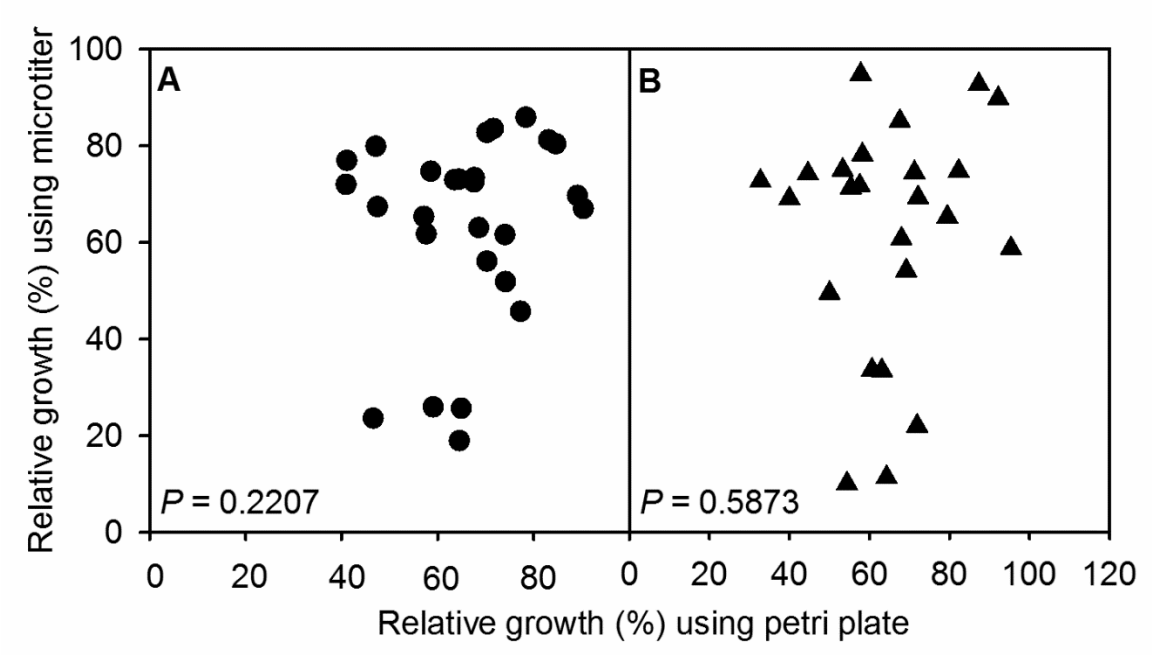

Figure D.2. Relationship between relative growth of *Venturia effusa* isolates to 3 µg/ml tebuconazole determined using a microtiter plate-based method and the traditional petri dish-based method. Values are the mean of replicates for each isolate. **A.** Experiment 1, **B.** Experiment 2.